

# Targeting Melanocortin Receptors as a Novel Strategy to Control Inflammation

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Abstract	2
I. Introduction	2
II. Proopiomelanocortin gene, gene expression, and post-translational processing	3
A. Proopiomelanocortin gene	3
B. Proopiomelanocortin gene expression	3
C. Post-translational processing of proopiomelanocortin	3
D. Melanocortin peptides	4
III. Melanocortin receptors and their endogenous antagonists	4
A. MC1 receptor	5
B. MC2 receptor	5
C. MC3 receptor	6
D. MC4 receptor	6
E. MC5 receptor	7
F. Agouti and agouti gene-related protein	7
IV. Intracellular signaling	8
V. Structure-activity relationship of melanocortin peptides	8
VI. Mechanism of the anti-inflammatory action of melanocortins	9
A. Receptor subtypes involved in the anti-inflammatory effects of melanocortins	9
B. Influence of melanocortins on nuclear factor- $\kappa$ B-mediated transcription	10
C. Melanocortins modulate production of chemical mediators of inflammation	11
1. Effects in vitro	11
2. Effects in vivo	12
D. Central control of peripheral inflammation	12
VII. Antipyretic influences of melanocortins	13
VIII. Changes in endogenous $\alpha$ -melanocyte-stimulating hormone in inflammatory disorders	15
IX. Potential therapeutic targets based on preclinical studies in inflammatory disorders	15
A. Acute inflammation	15
1. Allergic inflammation	15
2. Autoimmune uveoretinitis	17
3. Gouty arthritis	17
B. Chronic inflammatory diseases	17
1. Rheumatoid arthritis	17
2. Inflammatory bowel diseases	17
C. Inflammation within the brain and neurodegenerative disorders	18
D. Peripheral neuropathies	18
E. Systemic host reactions	19
1. Septic shock	19
2. Systemic vasculitis	20
3. Acute respiratory distress syndrome	20
4. Hemorrhagic shock	20
F. Ischemia and reperfusion injury	20
G. Organ transplantation	21

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H. Infections .....	21
X. Advantages over currently used anti-inflammatory drugs and potential disadvantages .....	23
References .....	24

**Abstract**—Adrenocorticotrophic hormone and  $\alpha$ -,  $\beta$ -, and  $\gamma$ -melanocyte-stimulating hormones, collectively called melanocortin peptides, exert multiple effects upon the host. These effects range from modulation of fever and inflammation to control of food intake, autonomic functions, and exocrine secretions. Recognition and cloning of five melanocortin receptors (MCRs) has greatly improved understanding of peptide-target cell interactions. Preclinical investigations indicate that activation of certain MCR subtypes, primarily MC1R and MC3R, could be a novel strategy to control inflammatory disorders. As a con-

sequence of reduced translocation of the nuclear factor  $\kappa$ B to the nucleus, MCR activation causes a collective reduction of the major molecules involved in the inflammatory process. Therefore, anti-inflammatory influences are broad and are not restricted to a specific mediator. Short half-life and lack of selectivity could be an obstacle to the use of the natural melanocortins. However, design and synthesis of new MCR ligands with selective chemical properties are already in progress. This review examines how marshaling MCR could control inflammation.

## I. Introduction

Adrenocorticotrophic hormone (ACTH) and  $\alpha$ -,  $\beta$ -, and  $\gamma$ -melanocyte-stimulating ( $\alpha$ -,  $\beta$ -,  $\gamma$ -MSH) hormones derive from post-translational processing of the precursor molecule proopiomelanocortin (POMC) (Eberle, 1988; Hadley and Haskell-Luevano, 1999) (Fig. 1). These POMC products are collectively called melanocortin peptides or melanocortins. Although adrenal stimulatory effects of ACTH and pigmentary influences of MSH have been known for over 50 years, the discovery that melanocortin peptides have multiple effects on the host is much more recent. These effects are disparate and range from modulation of fever and inflammation to control of food intake, autonomic functions, and exocrine secretions. Furthermore, recent research indicates that certain melanocortin peptides have antimicrobial effects.

Recognition and cloning of melanocortin receptors (MCRs) has greatly improved understanding of peptide-target cell interactions. Synthetic melanocortins with selective affinities for individual MCR may soon form

the basis for new classes of therapeutic molecules. In this article we summarize current physiological and pharmacological knowledge on melanocortins and their receptors and discuss possible therapeutic targets with a focus on treatment of inflammatory disorders. Treatment of obesity and sexual dysfunction are other significant therapeutic targets for receptor-specific melanocortins; for these topics we refer the readers to recent reviews (Beck, 2000; Wessells et al., 2000; Adan and Vink, 2001; Boston, 2001; Crowley et al., 2002; MacNeil et al., 2002; Van der Ploeg et al., 2002; Goodfellow and Saunders, 2003). A detailed description of effects of melanocortins in skin physiology and melanocyte function (Luger et al., 1997; Hadley et al., 1998; Slominski and Pawelek, 1998; Thody, 1999; Abdel-Malek et al., 2000; Bohm and Luger, 2000; Slominski and Wortsman, 2000; Slominski et al., 2000) and influences on behavior, learning, and memory (Beckwith et al., 1977, 1989; Datta and King, 1982; Klusa et al., 1998; Smolnik et al., 2000) are likewise beyond the scope of this review.

<sup>1</sup>Abbreviations: ACTH, adrenocorticotrophic hormone;  $\alpha$ -,  $\beta$ -,  $\gamma$ -MSH,  $\alpha$ -,  $\beta$ -,  $\gamma$ -melanocyte-stimulating hormones; POMC, proopiomelanocortin; MCR, melanocortin receptor;  $\beta$ -,  $\gamma$ -LPH,  $\beta$ -,  $\gamma$ -lipotropin; AgRP, agouti gene-related protein; STAT, signal transducers and activators of transcription; PC, precursor (or proprotein) convertase; PACE, furin; G protein, guanine nucleotide-binding protein; PKA, protein kinase A; MITF, microphthalmia-associated transcription factor; NF- $\kappa$ B, nuclear factor  $\kappa$ B; NDP- $\alpha$ -MSH, 4-norleucine, 7-D-phenylalanine- $\alpha$ -MSH; LPS, lipopolysaccharide; E-selectin, endothelial cell selectin; VCAM, vascular cell adhesion molecule; ICAM, intercellular adhesion molecule; TNF- $\alpha$ , tumor necrosis factor  $\alpha$ ; IL, interleukin; HP228, Ac-[Nle<sup>4</sup>,Gln<sup>5</sup>,D-Phe<sup>7</sup>,D-Trp<sup>9</sup>] $\alpha$ -MSH (4–10)-NH<sub>2</sub>; HS014, Ac-c[Cys,Glu,His,D-2'-Nal,Arg,Trp,Gly,Cys]Pro,Pro,Lys,Asp-NH<sub>2</sub>; HS059, cyclic [Ac-Cys<sup>3</sup>,Nle<sup>4</sup>,Arg<sup>5</sup>,D-2'-Nal<sup>7</sup>,Tyr<sup>10</sup>,Cys-NH<sub>2</sub>]<sup>11</sup> $\alpha$ -MSH (3–11); Melanotan II, Ac-Nle<sup>4</sup>-c[Asp<sup>5</sup>,D-Phe<sup>7</sup>,Lys<sup>10</sup>] $\alpha$ -MSH (4–10)-NH<sub>2</sub>; MS05, [H-Ser<sup>2</sup>,Ile<sup>3</sup>,Ile<sup>4</sup>,Ser<sup>5</sup>] $\alpha$ -MSH; ORG 2766, [Met(O<sub>2</sub>)<sup>4</sup>,D-Lys<sup>8</sup>,Phe<sup>9</sup>] $\alpha$ -ACTH (4–10); iNOS, inducible nitric oxide synthase; HIV, human immunodeficiency virus; gp120, glycoprotein 120; IFN- $\gamma$ ,  $\gamma$ -interferon;  $\alpha$ ,  $\alpha$ ; NO, nitric oxide; BAEP, brainstem auditory evoked potential.

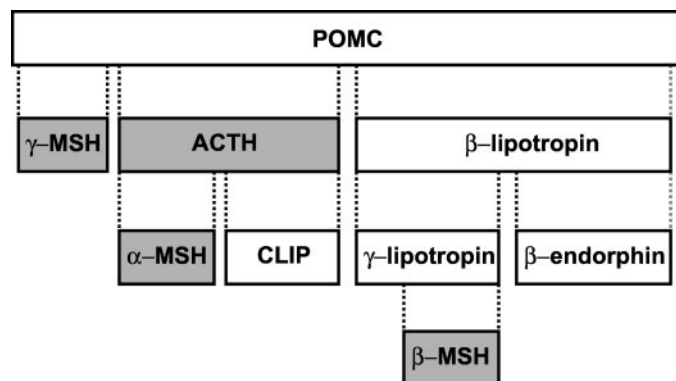


FIG. 1. Melanocortin peptides, ACTH and  $\alpha$ -,  $\beta$ -, and  $\gamma$ -MSH, derive from post-translational processing of POMC, which is also the precursor for opioid peptides and CLIP (corticotropin-like intermediate lobe peptide).

## II. Proopiomelanocortin Gene, Gene Expression, and Post-Translational Processing

### A. Proopiomelanocortin Gene

In 1977, a common precursor for ACTH/ $\alpha$ -MSH and  $\beta$ -lipotropin ( $\beta$ -LPH)/ $\beta$ -MSH/ $\beta$ -endorphin was found in mouse AtT-20 pituitary tumor cells (Mains et al., 1977; Roberts and Herbert, 1977). One year later, the molecule was demonstrated in a human nonpituitary tumor cell line (Bertagna et al., 1978). In 1979, analysis of the nucleotide sequence of cloned cDNA of bovine POMC (Nakanishi et al., 1979) showed a hitherto unknown MSH-like peptide sequence, called  $\gamma$ -MSH, as well as other N-terminal peptides. Gene sequences for human (Chang et al., 1980) and rat (Drouin and Goodman, 1980) POMC were described shortly thereafter. POMC sequence was subsequently determined in several species of mammals, amphibians, and teleosts; all the sequences revealed the same structural organization. In humans, there is a single POMC gene per haploid nucleus located on chromosome 2p23. The pituitary of lamprey, the most ancient of vertebrates, contains recognizable POMC sequences with structural similarity to those of teleosts and higher vertebrates, suggesting that POMC was present in common ancestors of lampreys and gnathostomes some 700 million or more years ago (Heinig et al., 1995). In humans, the POMC gene is unusual in that it possesses three promoter regions that control transcription: promoter 1 (P1), P2, and P3 (Kraus et al., 1993). P1 is the predominant promoter in some cancers; P2 controls transcription in the normal pituitary gland; and P3 is weakly active in a variety of peripheral tissues (Kraus et al., 1993).

### B. Proopiomelanocortin Gene Expression

In addition to the pituitary, where it was originally found, POMC expression and peptide processing occur normally in the nervous system and in widespread peripheral tissues. In the brain, POMC cell bodies are found in the hypothalamic arcuate nucleus and nucleus of solitary tract in the caudal brainstem (Cone et al., 2001; Pritchard et al., 2002). POMC mRNA is also detectable in the spinal cord and dorsal root ganglion (Plantinga et al., 1992; van der Kraan et al., 1999). Within the hypothalamus, the integrating center for energy balance, POMC neurons have extensive interactions with other pathways. Melanocortinergic terminals are found in various hypothalamic regions such as paraventricular, dorsomedial hypothalamic nucleus, arcuate nucleus, and lateral hypothalamic regions (Bagnol et al., 1999). The POMC neurons in the arcuate nucleus express leptin receptors, through which leptin regulates POMC expression (Schwartz et al., 1997; Elmquist et al., 1999). The arcuate nucleus POMC neurons also express neuropeptide Y1 and Y5 receptors, receive neuropeptide Y innervations, and interact with neuropeptide Y/agouti gene-related protein (AgRP) neurons locally (Broberger

et al., 1998; Bagnol et al., 1999). POMC neurons project broadly to many brain regions, including those hypothalamic and brainstem nuclei important for regulating energy homeostasis. Furthermore, POMC neurons send projections to sympathetic preganglionic neurons in the thoracic spinal cord.

Although ectopic POMC syndrome associated with malignancies has been known for decades (Beuschlein and Hammer, 2002) knowledge that POMC is expressed also in normal tissues is more recent (Blalock, 1985, 1999). Especially important to inflammation, POMC mRNA also occurs in lymphocytes, monocytes, keratinocytes, and melanocytes, and it is clear that POMC peptides have regulatory functions in these cells (Star et al., 1995; Chakraborty et al., 1996; Blalock, 1999; Slominski and Wortsman, 2000; Slominski et al., 2000). There is evidence that leukemia inhibitory factor stimulates POMC expression via phosphorylation of signal transducers and activators of transcription (STATs) STAT1 and STAT3 proteins (Ray et al., 1996). Therefore, activation of the STAT signaling pathway by cytokines, interferons, or hormones can increase POMC expression and melanocortin peptide production at sites of infection or inflammation. It appears that there are multiple forms of POMC transcripts. Pituitary POMC mRNA encodes a secreted protein, whereas certain brain and peripheral cells express a truncated POMC mRNA without coding for a signal sequence (Farooqui et al., 1995; Millington et al., 1999).

### C. Post-Translational Processing of Proopiomelanocortin

Biologically active proteins and peptides are often generated by intracellular proteolysis of inactive precursors (Seidah et al., 1999). This evolutionarily ancient mechanism depends on the production of specific secretory enzymes and the tight regulation of their activities. Such processing enzymes usually cleave proproteins at selected sites composed of single or paired basic amino acids. The latter are found in precursors of most of neural and peptide hormones, proteolytic enzymes, growth factors receptors, and signaling molecules (Seidah et al., 1991). Thus, generation of biologically active peptides and proteins depends on two main components: the polypeptide precursor substrate and the proteolytic enzyme(s) responsible for the conversion of the precursor into its final bioactive protein-peptide product.

The secretory enzymes responsible for intracellular cleavage of POMC have been characterized. They belong to a family of serine proteinases of the subtilisin/kexin-type (Seidah and Chretien, 1994). There are seven known mammalian precursor (or proprotein) convertases (PCs) cleaving at single and/or pairs of basic residues: PC1 (also called PC3), PC2, furin (also called PACE), PACE4, PC4, PC5 (also called PC6), and PC7 (also called SPC7, LPC, or PC8) (Seidah and Chretien, 1994; Rouillé et al., 1995; Seidah et al., 1999). PCs show



a remarkable temporal and spatial specificity of expression patterns that make them available in various proportions and combinations in different cell types. Conservation of the catalytic domains and variability of these domains suggest that PC genes evolved from a common ancestral gene. Except for furin and PACE4, which are closely linked, all the PC genes are dispersed on various chromosomes. Recent research indicates that although many PCs could cleave the same precursor in vitro, in vivo processing depends on regulation of individual cellular expression and activity as well as intraorganellar localization, which are critical for each processing reaction (Seidah et al., 1999).

Analysis of tissue expression and cellular localization of the convertases showed that only PC1 and PC2 are found in dense secretory granules. These enzymes have a key role in the processing of neuropeptide and endocrine precursors whose products are stored in granules (Malide et al., 1995). All the other enzymes concentrate and act at the level of the *trans*-Golgi network, en route to the cell surface, or at the level of plasma membrane (Seidah et al., 1992, 1996; Seidah and Chretien, 1994). Thus, the latter group of enzymes appears to be primarily responsible for processing precursors whose products reach the cell surface or are secreted constitutively.

When PC1 and PC2 were discovered, POMC was soon identified as a potential substrate. This idea was supported by the observation of enhanced expression of such enzymes in pituitary POMC-producing cells. Data showed that PC1 generated ACTH and  $\beta$ -LPH, whereas PC2 was required for production of  $\alpha$ -MSH and  $\beta$ -endorphin (Benjannet et al., 1991). This was the first evidence that tissue-specific processing of POMC could be explained by the relative expression of its convertases. Thus, in the corticotrophs where PC1 predominates, ACTH and  $\beta$ -LPH are the final POMC-processing products. In contrast, expression of PC2 in the pituitary pars intermedia accounts for production of  $\alpha$ -MSH and  $\beta$ -endorphin (Day et al., 1992; Marcinkiewicz et al., 1993). PACE4 and furin can also generate ACTH and  $\beta$ -LPH both in the pituitary and in extrapituitary sites. Indeed, it is clear that POMC, PC1, and PC2, as well as other

convertases, are expressed in extrapituitary tissues including the immune system (Blalock, 1985) and the skin (Wakamatsu et al., 1997).

#### D. Melanocortin Peptides

Amino acid sequences of human melanocortin peptides are shown in Fig. 2.  $\alpha$ - and  $\beta$ -MSH and ACTH were purified and sequenced in the 1950s (Eberle, 1988).  $\alpha$ -MSH was found to share the sequence of ACTH (1–13), although  $\alpha$ -MSH is acetylated at the N terminus and C-terminally amidated. The structure of the  $\beta$ -MSH peptides of different vertebrates is more variable than that of  $\alpha$ -MSH (Eberle, 1988).  $\beta$ -MSH was originally isolated from human pituitaries as a side fraction of somatotropin preparation (Dixon, 1960) and thereafter found to correspond to the 37–58 region of human  $\beta$ -LPH (Li and Chung, 1976). Subsequently, the peptide was considered to be an artifact of procedure and did not exist in humans. However, more recently, naturally occurring  $\beta$ -MSH-octadecapeptide has been identified in human hypothalamus (Bertagna et al., 1986). The peptide corresponds with the  $\gamma$ -LPH (41–58) sequence. Subsequently, POMC was found to contain yet another MSH peptide sequence,  $\gamma$ -MSH. All the melanocortin peptides share an “invariant” sequence of four amino acids, His-Phe-Arg-Trp, which are the residues 6–9 in ACTH and  $\alpha$ -MSH.

### III. Melanocortin Receptors and Their Endogenous Antagonists

The five MCRs cloned so far belong to the class A of guanine nucleotide-binding protein (G protein)-coupled, seven transmembrane receptors (Cone, 2000). They are the product of small genes, many of which are polymorphic. The MCRs show high sequence homologies, ranging from 60% identity between MC4R and MC5R, to 38% identity between MC2R and MC4R. MCRs are the smallest G protein-coupled receptors known, with short amino- and carboxyl-terminal ends and a very small second extracellular loop (Fig. 3). All are functionally coupled to adenylyl cyclase and mediate their effects primarily by activating a cAMP-dependent signaling pathway.

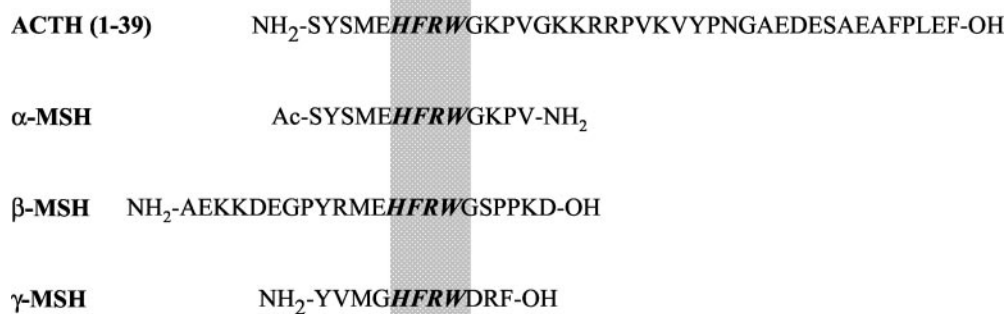


FIG. 2. Amino acid sequence of melanocortin peptides. All the melanocortins share the invariant sequence HFRW.

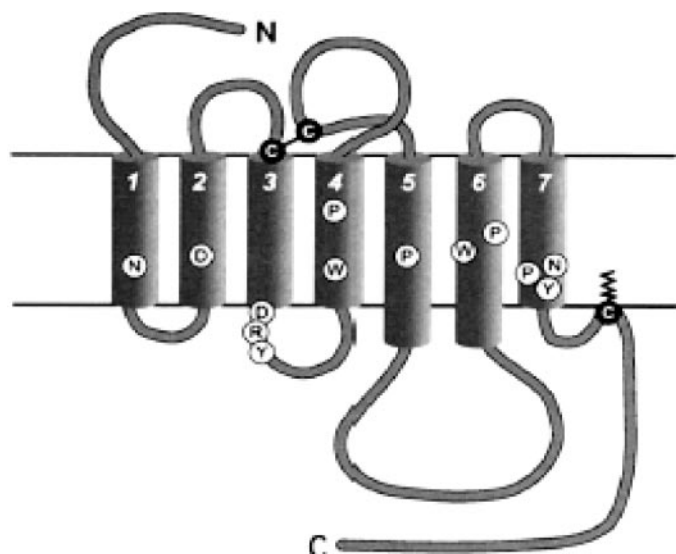


FIG. 3. The MCRs belong to the class A of G protein-coupled seven transmembrane receptors (rhodopsin/ $\beta_2$ -adrenergic-like family), which also includes biogenic amine, cannabinoid, melatonin, chemokine, and several other receptors. MCRs share many features with other G protein-coupled receptors: they have several potential *N*-glycosylation sites in their amino-terminal domains, consensus recognition sites for protein kinases C and/or A, which indicate that they may undergo regulation by phosphorylation, and conserved cysteines in their carboxyl termini, potential sites for fatty acid acylation, anchoring the C-terminal end to plasma membrane.

MCRs share many features with other G protein-coupled receptors (Rana, 2003): they have several potential *N*-glycosylation sites in their amino-terminal domains, consensus recognition sites for protein kinase C and/or A (PKA), and conserved cysteines in their carboxyl termini (Wikberg et al., 2000; Abdel-Malek, 2001). Ligand affinity, tissue distribution, and functions of each receptor subtype are reported in Table 1.

#### A. MC1 Receptor

MC1R was the first member of the MCR gene family to be cloned (Chhajlani and Wikberg, 1992; Mountjoy et al., 1992). The cloned cDNA encoded a 317-amino acid protein with the transmembrane topography characteristic of receptors that couple to heterotrimeric G proteins. The relative affinity of the human MC1R for the natural melanocortins is  $\alpha$ -MSH  $\geq$  ACTH  $>$   $\beta$ -MSH  $\gg$   $\gamma$ -MSH (Chhajlani and Wikberg, 1992; Suzuki et al., 1996). These differences in affinity reproduce the relative potency of the melanocortin peptides in stimulation of melanogenesis and explain the lack of melanogenic activity of  $\gamma$ -MSH (Abdel-Malek, 2001).

$\alpha$ -MSH/MC1R interactions contribute to regulation of skin physiology and melanogenesis (Slominski et al., 2000). Binding of  $\alpha$ -MSH to its MC1R in melanocytes starts a signal cascade that activates adenylyl cyclase, increases intracellular cAMP, and induces activity of tyrosinase, the rate-limiting enzyme in the eumelanin synthetic pathway (Hunt et al., 1994; Abdel-Malek et al., 1995). MC1R mRNA in the skin is up-regulated by its

own melanocortin ligands and by endothelin-1 (Abdel-Malek, 2001). Furthermore, MC1R expression appears to be regulated by the microphthalmia-associated transcription factor (MITF). This transcription factor belongs to the family of bHLH-LZ type transcription factors and promotes transcription of genes for melanogenesis-related enzymes such as tyrosinase. Although promoter deletion and transactivation studies failed to demonstrate direct MC1R activation by MITF through this site (Smith et al., 2001), in coexpression studies, induction of MC1R promoter activity increased by 5-fold in the presence of MITF (Aoki and Moro, 2002). In addition to effects in melanocytes, there is evidence that MITF enhances expression of MC1R in cultured murine mast cells (Adachi et al., 2000).

It is clear that MC1R functions extend well beyond regulation of melanogenesis. MC1R expression occurs in macrophage/monocytic cells (Star et al., 1995; Bhardwaj et al., 1997; Taherzadeh et al., 1999), lymphocytes with antigen-presenting and cytotoxic functions (Neumann Andersen et al., 2001), neutrophils (Catania et al., 1996), endothelial cells (Hartmeyer et al., 1997), astrocytes (Wong et al., 1997), and fibroblasts (Bohm et al., 1999b). Peripheral blood-derived dendritic cells were likewise found to express MC1R (Becher et al., 1999; Salazar-Onfray et al., 2002). Although this receptor subtype occurs mainly in peripheral tissues, *in situ* hybridization and immunohistochemistry techniques demonstrated its expression also in scattered neurons of periaqueductal gray substance in rat and human brains (Xia et al., 1995). Transactivation of MC1R in inflammatory cells causes marked reduction of activation and translocation to the nucleus of the transcription factor NF- $\kappa$ B (Manna and Aggarwal, 1998). Consequently, there are marked anti-inflammatory effects exerted through inhibition of NF- $\kappa$ B-mediated transcription (see below).

Flow cytometry studies showed that MC1R is expressed by *in vitro*-activated monocytes/macrophages and by the THP-1 monocytic cell line, at ratios of approximately one third to one fifth that of melanoma cells. However, although MC1R in immunocytes and endothelial cells is activated by picomolar concentration of  $\alpha$ -MSH, MC1R activation in melanocytes requires nanomolar concentrations of the peptide (Suzuki et al., 1996; Kalden et al., 1999; Scholzen et al., 1999). Therefore, although receptor density in inflammatory cells is less than that in melanocytes, it appears that receptor affinity is much greater.

#### B. MC2 Receptor

The melanocortin-2 receptor (MC2R), also known as ACTH receptor, is selectively activated by adrenocorticotrophic hormone. The ACTH receptor/MC2R gene was originally isolated by homology screening of human cDNA and genomic DNA libraries. The ACTH receptor gene encodes a 297-amino acid G protein-coupled recep-

TABLE 1  
Affinity, distribution, and functions of MCR subtypes

MCR Subtype	Ligand Affinity	Prevalent Tissue Expression	Functions
MC1R	$\alpha$ -MSH $\geq$ ACTH $\gg$ $\gamma$ -MSH	Melanocytes Immune/inflammatory cells; keratinocytes; endothelial cells; glial cells	Pigmentary effects Antipyretic/anti-inflammatory
MC2R	ACTH	Adrenal cortex	Steroidogenesis
MC3R	$\gamma$ -MSH = ACTH $\geq$ $\alpha$ -MSH	CNS Macrophages	Autonomic functions Anti-inflammatory
MC4R	$\alpha$ -MSH = ACTH $\gg$ $\gamma$ -MSH	CNS	Control of feeding and energy homeostasis; erectile activity
MC5R	$\alpha$ -MSH $\geq$ ACTH $>$ $\gamma$ -MSH	Exocrine glands, lymphocytes	Regulation of exocrine secretions, immunoregulatory functions

CNS, central nervous system.

tor and shows the characteristic seven transmembrane-spanning domains that form the ligand binding site.

The physiological influences of ACTH on production and release of steroids by the adrenal cortex, their circadian variation, and stress-related fluctuations are mediated by MC2R (Lefkowitz et al., 1970; Buckley and Ramachandran, 1981; Mountjoy et al., 1992). Binding of ACTH to its receptor stimulates adenylyl cyclase and induces increases in cell cAMP; this leads to activation of PKA, which promotes expression of steroidogenic enzymes (Penhoat et al., 1989).

In situ hybridization studies revealed dense expression of MC2R (Mountjoy et al., 1992; Xia and Wikberg, 1996) in the zona glomerulosa and zona fasciculata of the adrenal cortex, the sites of mineralocorticoid and glucocorticoid production (Mountjoy et al., 1992; Liakos et al., 1998). The zona reticularis showed less mRNA labeling. In the adrenal medulla, only scattered cells with unknown functions stain for MC2R mRNA (Xia and Wikberg, 1996). MC2R expression in adrenal cells is up-regulated by its ligand ACTH (Mountjoy et al., 1994a).

In addition to the adrenal glands, the MC2R mRNA has been found in murine adipocytes (Boston and Cone, 1996; Cammas et al., 1997), where it is believed to mediate stress-induced lipolysis in response to ACTH (Boston, 1999). However, ACTH does not appear to regulate adipocyte function in humans and other primates, as human adipocytes lack expression of MC2R (Chhajlani, 1996). Furthermore, recent research indicated failure to export the ACTH receptor from the endoplasmic reticulum in nonadrenal cells, suggesting requirement for a specific adrenal accessory factor (Noon et al., 2002).

### C. MC3 Receptor

The MC3R gene encodes a G protein-linked receptor, coupled to both cAMP- and inositol phospholipid- $\text{Ca}^{2+}$ -mediated signaling systems (Konda et al., 1994). Polymerase chain reaction primed with degenerated oligonucleotides, whose sequence was based on the homologous transmembrane regions of the other seven transmembrane G protein-linked receptors, identified a third member of the melanocortin receptor family that recognizes the core heptapeptide sequence of melanocortins

(Gantz et al., 1993a). The MC3R is the only MCR activated by  $\gamma$ -MSH with potency similar to that of other melanocortins ( $\gamma$ -MSH = ACTH  $\geq$   $\alpha$ -MSH) (Roselli-Rehfuß et al., 1993). This intronless gene encodes a protein of 361 amino acids.

MC3R expression occurs in brain, placenta, and gut but not in melanoma cells or in the adrenal gland (Gantz et al., 1993a). MC3R expression also occurs in the heart (Chhajlani, 1996), in human monocytes (Taherzadeh et al., 1999), and in mouse peritoneal macrophages (Getting et al., 1999). A map of MC3R expression in the brain obtained by in situ hybridization showed abundant presence in the hypothalamus and limbic system, but signals for this receptor were also present in the septum, thalamus, hippocampus, and midbrain (Roselli-Rehfuß et al., 1993). POMC neurons of the rat arcuate nucleus were found to express mRNA for MC3R (Jegou et al., 2000).

MC3R appears to participate in modulation of autonomic functions, feeding, and inflammation (Abdel-Malek, 2001; Getting, 2002). Hypotension and bradycardia elicited by the release of  $\alpha$ -MSH from the arcuate neurons appear to be mediated by MC3R and MC4R located in the medullary dorsal-vagal complex (Li et al., 1996). Participation of MC3R in energy homeostasis was disclosed in MC3R-deficient mice, which showed increased fat mass, reduced lean mass, and higher ratio of weight gain to food intake (Chen et al., 2000). Recent data suggest that MC3R activation mediates protective influences of melanocortins in myocardial ischemia/reperfusion-induced arrhythmias in rats (Guarini et al., 2002). Furthermore, activation of MC3R has clear anti-inflammatory influences (Getting et al., 1999, 2002, 2003a; Getting and Perretti, 2000).

### D. MC4 Receptor

The human MC4R was the second neural MCR to be cloned. Its affinity for the melanocortins has certain similarities with that of MC1R. The order of potency for activation of MC4R is  $\alpha$ -MSH = ACTH  $>$   $\beta$ -MSH  $\gg$   $\gamma$ -MSH.

MC4R is a 332-amino acid protein encoded by a single exon of 999 nucleotides. The rat homologous gene is 93%



identical to the human gene (Alvaro et al., 1996), which suggests that the gene is highly conserved in mammals.

By the use Northern blot analysis and in situ hybridization techniques, MC4R was found primarily in the brain (Gantz et al., 1993b). The distribution of this receptor in the central nervous system is much broader than that of MC3R and includes the cortex, the thalamus, the hypothalamus, the brainstem, and the spinal cord (Mountjoy et al., 1994b). Conversely, the MC4R was not detected in peripheral cells in an extensive study including 20 different tissues (Chhajlani, 1996).

Distribution of MC4R is consistent with its involvement in autonomic and neuroendocrine functions. Evidence that this receptor subtype regulates food intake and energy expenditure is based on gene-targeting in mice, which results in maturity-onset obesity, with hyperphagia, hyperinsulinemia, and hyperglycemia (Huszar et al., 1997). Homozygous MC4R-deficient mice do not respond to the anorectic effects of  $\alpha$ -MSH. It appears, therefore, that  $\alpha$ -MSH inhibits food intake through activation of MC4R (Marsh et al., 1999). Mice with MC4R deficiency have enhanced caloric efficiency, similar to that observed in the agouti obesity syndrome and in the MC3R-null mice (Ste Marie et al., 2000). Mice lacking both MC3R and MC4R are significantly heavier than those deficient in MC4R only, suggesting that the two receptors serve nonredundant functions in the regulation of energy homeostasis (Chen et al., 2000). Recent research indicates that MC4R modulates erectile function and sexual behavior, possibly through neuronal circuitry in spinal cord erectile centers and somatosensory afferent nerve terminals of the penis (Wessells et al., 1998; Van der Ploeg et al., 2002).

#### E. MC5 Receptor

The melanocortin-5 receptor (MC5R) is similar to the MC1R and MC4R in its capacity to recognize  $\alpha$ -MSH and ACTH but not  $\gamma$ -MSH ( $\alpha$ -MSH  $\geq$  ACTH  $\gg$   $\gamma$ -MSH). MC5R contributes to regulation of exocrine gland function and to certain immune responses.

The MC5R was the last of the MCR gene family to be cloned by homology screening from genomic DNA in man (Chhajlani et al., 1993), mouse (Labbe et al., 1994), and rat (Griffon et al., 1994). The human gene encodes for a protein of 325 amino acids.

MC5R is ubiquitously expressed in peripheral tissues. It occurs in the adrenal glands, fat cells, kidney, liver, lung, lymph nodes, bone marrow, thymus, mammary glands, testis, ovary, pituitary testis, uterus, esophagus, stomach, duodenum, skin, lung, skeletal muscle, and exocrine glands (Gantz et al., 1994; Labbe et al., 1994; Fathi et al., 1995; Chhajlani, 1996; Chen et al., 1997; van der Kraan et al., 1998; Colombo et al., 2002). Presence of MC5R in B- and T-lymphocytes suggests a function in immune regulation. Indeed, recent data suggest that  $\alpha$ -MSH participates in B-lymphocyte function via the activation of the Jak/STAT pathway, the intracellu-

lar phosphorylation pathway used by cytokines and growth factors, through specific binding to the MC5R (Buggy, 1998). Furthermore,  $\alpha$ -MSH can induce CD25<sup>+</sup> CD4<sup>+</sup> regulatory T cells through the MC5R expressed on primed T cells (Taylor and Namba, 2001).

Targeted disruption of the MC5R gene produced mice with a severe defect in water repulsion and thermoregulation caused by decreased production of sebaceous lipids (Chen et al., 1997). High expression of MC5R occurs in multiple exocrine tissues, and the receptor is required for production of porphyrins by the Harderian gland and for protein and tear secretion by the lacrimal gland (Entwistle et al., 1990; Chen et al., 1997). These data suggest a coordinated system for regulation of exocrine gland function by melanocortin peptides; also, that the MC5R is the mediator of the sebostrophic activity of  $\alpha$ -MSH described in early studies (Thody and Shuster, 1970, 1975).

#### F. Agouti and Agouti Gene-Related Protein

Two melanocortin receptor antagonists, the agouti (also termed agouti signaling protein) and AgRP, participate in control of melanocortin signaling (Ollmann et al., 1998; Wikberg et al., 2000). Both agouti and AgRP contain cysteine-rich C-terminal domains that form disulfide bridges leading to similar folded structures (Dinulescu and Cone, 2000). The entire antagonistic activity for MCRs resides in the Cys-rich end of the molecule (Willard et al., 1995). The agouti was described as a genetic locus controlling skin pigmentation long before it was cloned (Seechurn et al., 1988). In rodents, the agouti consists of a 131-amino acid protein, showing characteristics of a secreted protein with a hydrophobic signal sequence, which is expressed in skin only (Bultman et al., 1992; Lu et al., 1994). The human agouti is a protein closely homologous to the rodent agouti, but it shows a much wider distribution as it is expressed in adipose tissue, testis, ovary, heart, and, at lower levels, in foreskin, kidney, and liver (Wilson et al., 1995; Voisey and van Daal, 2002). Agouti is a competitive antagonist at melanocortin receptors with high affinity at MC1R (Blanchard et al., 1995), although it also shows antagonistic activity for the human MC4R. This receptor antagonist may be important in inflammatory responses. Indeed, mice carrying the dominant agouti allele lethal yellow showed greater acute inflammatory responses than control animals (Lipton et al., 1999).

The AgRP, a competitive antagonist for MC3R and MC4R, was cloned on the basis of its homology to agouti (Ollmann et al., 1997). The AgRP shows a very distinct expression in the central nervous system, as it is expressed in neural cell bodies of posterior hypothalamus in close vicinity to the POMC-expressing neurons (Broberger et al., 1998). AgRP-containing neurons project to many of the same hypothalamic nuclei that receive projections from POMC neurons. The POMC and AgRP systems may function as physiologically opposing sys-

tems, where the former decreases the drive for feeding and the latter increases it (Wilson et al., 1999; Wirth and Giraud, 2000).

#### IV. Intracellular Signaling

The transmembrane signaling of melanocortin peptides involves stimulation of adenylyl cyclase followed by synthesis of cAMP, which induces activation of protein kinase(s) and protein phosphorylation (Eberle et al., 1978; Eberle, 1988). The first report that adenylyl cyclase is involved in mediating effects of MSH appeared in 1965 (Bitensky and Burstein, 1965). Subsequently, many investigators have shown that signaling of melanocortin peptides arises via activation of adenylyl cyclase and elevation of cAMP. Stimulation of cAMP production by the MCRs causes activation of PKA, the catalytic subunit of which phosphorylates the cAMP response element-binding protein which then binds to cAMP response elements in the DNA (Busca and Ballotti, 2000; Harris et al., 2001; Sarkar et al., 2002).

Ca<sup>2+</sup> plays a key role in MSH-receptor binding and signal transduction, since both the affinity of the ligand to the receptor and the signaling are markedly enhanced under physiological concentrations of extracellular Ca<sup>2+</sup>, relative to transduction and binding under Ca<sup>2+</sup>-free conditions (Gerst et al., 1987). Binding of  $\beta$ -MSH to B16-M2R is minimal in Ca<sup>2+</sup>-free (<50 nM) medium, reaches a first plateau between 1 and 5  $\mu$ M Ca<sup>2+</sup>, and is maximal at 1 mM Ca<sup>2+</sup>. However, although Ca<sup>2+</sup> is required for MSH-receptor binding at low peptide concentrations (1–10 nM), it is not essential at high peptide concentrations (50–500 nM). Calmodulin inhibitors inhibit  $\beta$ -MSH-receptor binding as well as the subsequent stimulation of adenylyl cyclase (Gerst and Salomon, 1988). This observation suggests that a calmodulin-related Ca<sup>2+</sup>-binding protein regulates binding to the receptor. Therefore, melanocortin peptides belong to the class of peptide hormones whose receptors require extracellular Ca<sup>2+</sup> for hormone binding and signal transduction.

The affinity of MSH-peptides for their receptors is not only modulated by Ca<sup>2+</sup> but also by GTP. Guanosine nucleotides decrease MSH-receptor binding and induce dissociation of preformed MSH-receptor complex in a calcium-independent manner (Rodbell, 1980; Gerst et al., 1987).

#### V. Structure-Activity Relationship of Melanocortin Peptides

Basic information about structural requirements for specific functions of melanocortins was initially obtained by comparing activity profiles of naturally occurring peptides in different assays. Such comparative studies mainly evaluated melanotropic activity of each natural peptide (Hruby et al., 1987; Eberle, 1988). Subsequent research was focused on synthetic analogs and frag-

ments of  $\alpha$ -MSH and other melanocortin peptides. In 1980, synthesis of 4-norleucine, 7-D-phenylalanine- $\alpha$ -MSH, (Nle<sup>4</sup>,D-Phe<sup>7</sup>)- $\alpha$ -MSH, produced a superpotent analog of the natural peptide that has been largely used since in research on  $\alpha$ -MSH (Sawyer et al., 1980). After recognition and cloning of melanocortin receptors, the principal aim was to design receptor-selective ligands with precise characteristics that could be useful for medical purposes. Binding assays and cAMP generation in cells transiently expressing MC1R, MC2R, MC3R, MC4R, and MC5R have improved knowledge of chemical properties that alter selectivity for each MCR subtype. Systematic amino acid substitutions were very important to design compounds that recognize specific receptor subtypes (Haskell-Luevano et al., 1996; Schioth et al., 1997a, 2002; Wikberg et al., 2000; Grieco et al., 2002; Kavarana et al., 2002; Han et al., 2003). To obtain selective compounds, it is seemingly important to identify substitutions that reduce binding for each of the receptors. Finally, mutations of receptor proteins and molecular modeling of both ligand and receptor structure have improved information on ligand binding requirements (Haskell-Luevano et al., 1996; Yang et al., 1997; Prusis et al., 2001; Holder and Haskell-Luevano, 2003).

Over the last decade, many investigations have explored structure-activity relations of melanocortins (Hruby and Han, 2000). An inactivation study based on alanine substitutions determined the relative importance of each amino acid in the  $\alpha$ -MSH sequence in binding activity of  $\alpha$ -MSH to human MC1R and rat MC3R (Sahm et al., 1994). This Ala-scan showed the importance of the amino acids in position 4–10 for binding to both these receptors (Sahm et al., 1994). For binding to MC1R, Met<sup>4</sup> appeared to be the most important amino acid outside the sequence 6–9. When this amino acid was replaced by Ala, there was a marked reduction in binding affinity for MC1R (Sahm et al., 1994). Further investigations showed that introduction of Asp in position 4 reduced binding to all MCRs, and particularly to MC3R (Schioth et al., 2002). His in position 6 has specific importance for binding to MC1R (Sahm et al., 1994; Schioth et al., 1997b, 2002). Another structure-activity study focused on a tetrapeptide library, based upon the template Ac-His-D-Phe-Arg-Trp-NH<sub>2</sub>. Peptides that had been modified at the Trp<sup>9</sup> position were characterized for agonist activity at the mouse melanocortin receptors MC1R, MC3R, MC4R, and MC5R (Holder et al., 2002). Results from this study showed that modification of the Trp<sup>9</sup> in the tetrapeptide template resulted in only small changes in potency at MC1R, whereas amino acid substitutions caused up to a 9700-fold decrease in potency at MC4R and MC5R. These observations suggest that MC1R is more tolerant to modifications in the invariant sequence. Another observation from this study is that the Trp<sup>9</sup> indole moiety in the tetrapeptide template is important for the MC3R agonist potency. This position could be used to design



melanocortin ligands possessing receptor selectivity for the predominantly peripheral MC1 and MC5 relative to the centrally expressed MC3 and MC4 receptors. Indeed, potency of the Ac-His-D-Phe-Arg-Tic-NH<sub>2</sub> and the Ac-His-D-Phe-Arg-Bip-NH<sub>2</sub> tetrapeptides was in the nanomolar range at MC1R and MC5R but in micromolar range at MC3R and MC4R (Holder et al., 2002).

C-terminally modified analogs of  $\alpha$ -MSH indicated the importance of Pro<sup>12</sup> for binding and activity at the MC1R (Peng et al., 1997). When Pro<sup>12</sup> in the  $\alpha$ -MSH sequence was substituted with Phe<sup>12</sup>, the potency of the peptide was slightly reduced. Furthermore, when Phe<sup>12</sup> was associated with Asp<sup>10</sup>, the affinity for MC1R of the peptide (Asp<sup>10</sup>, Phe<sup>12</sup>)- $\alpha$ -MSH was reduced to 0.069% and activity to 0.009, resulting in a virtually inactive peptide. An important observation in this research was that modifications of the melanocortin peptide sequence led to analogs for which either affinity or activity, but not both, were significantly altered (Peng et al., 1997). Therefore, the data confirmed the initial observations that binding to melanocortin receptors and their activation do not depend upon the same structure (Eberle, 1988). Synthesis of (Nle<sup>4</sup>,D-Phe<sup>7</sup>)- $\alpha$ -MSH analogs where the N- or C-terminal amino acids were deleted or substituted showed that the N-terminal segment (Ser<sup>1</sup>-Tyr<sup>2</sup>-Ser<sup>3</sup>) of (Nle<sup>4</sup>,D-Phe<sup>7</sup>)- $\alpha$ -MSH is not important for binding to MC1R or MC4R, whereas it does influence binding to MC3R and MC5R (Schioth et al., 1998). The C-terminal segment (Gly<sup>10</sup>-Lys<sup>11</sup>-Pro<sup>12</sup>-Val<sup>13</sup>) is important for binding to all four MCR subtypes.

The aromatic residues 1, 6, 8, and 11 and the basic residue Arg<sup>10</sup> are the essential residues for selectivity of  $\gamma$ -MSH for MC3R over MC4 and MC5 receptor subtypes (Grieco et al., 2000). A recent study shows the importance of the His-Phe-Arg-Trp sequence in receptor binding and in agonistic activity of  $\gamma$ -MSH (Grieco et al., 2002). The last four amino acids in the C-terminal region of  $\gamma$ -MSH are not important determinants of biological activity and selectivity at human melanocortin receptors, whereas the His-Phe-Arg-Trp sequence is relevant for activity.

Although major advances in the design and synthesis of more potent and selective MCR ligands have been made, there are still problems that need to be solved. One is that synthetic agonists or antagonists may encounter difficulties in reaching their target(s). For example, MC4R is located within the brain and, therefore, ligands must penetrate the blood-brain barrier to exert their effects. This important issue must be addressed before any new MC4R-targeted molecule could be considered for clinical use. The most widely used MC4R agonist is the cyclic lactam analog of  $\alpha$ -MSH Melanotan II, which penetrates the blood-brain barrier (Al-Obeidi et al., 1989). On the other hand, reduced accessibility to central receptors could be advantageous for molecules designed to act solely in the periphery. For instance, anti-inflammatory molecules that act exclusively in the periphery should circumvent the anorectic influences of MC4R activation.

## VI. Mechanism of the Anti-Inflammatory Action of Melanocortins

The anti-inflammatory influences of  $\alpha$ -MSH and other melanocortins are exerted through inhibition of inflammatory mediator production and inflammatory cell migration (Table 2). These influences occur through binding of melanocortins to melanocortin receptors on immunocytes and via descending anti-inflammatory neural pathways induced by stimulation of  $\alpha$ -MSH receptors within the brain (Lipton and Catania, 1997).

### A. Receptor Subtypes Involved in the Anti-Inflammatory Effects of Melanocortins

Melanocortin peptides exert anticytokine and anti-inflammatory effects in blood cells, cells of the immune system, and in other cell types including neural, endothelial, and epithelial cells. Although these influences are mainly exerted via activation of the known melanocortin receptors, it appears that there are other, still unknown mechanisms.

TABLE 2  
*Mechanism of the anti-inflammatory effect of melanocortins*

Effect	Target Cell, Tissue, or Organ	Reference
Reduced of production/expression of Proinflammatory cytokines and chemokines	Macrophages, endothelial cells, keratinocytes, fibroblasts, whole blood, liver	Cannon et al., 1986; Chiao et al., 1996, 1997; Lipton and Catania, 1997; Catania et al., 2000a; Luger et al., 2000; Moustafa et al., 2002
Nitric oxide (NO)	Macrophages, microglia, melanocytes, keratinocytes	Star et al., 1995; Delgado et al., 1998; Altavilla et al., 2000; Gupta et al., 2000; Haycock et al., 2000; Tsatmali et al., 2000
Oxygen peroxide Adhesion molecules (ICAM, VCAM)	Keratinocytes, melanocytes Endothelial cells, kidney, liver, heart	Haycock et al., 2000 Chiao et al., 1996, 1997; Luger et al., 2000; Gatti et al., 2002; Scholzen et al., 2003
Inhibition of white cell migration	Skin, lung, heart, kidney, liver, joints	Mason and Van Epps, 1989; Lipton et al., 1994; Catania et al., 1996; Chiao et al., 1996, 1997; Delgado Hernandez et al., 1999; Gatti et al., 2002; Getting et al., 2002

A major question concerns which melanocortin receptor subtypes are involved in the anti-inflammatory influences. Virtually all the cells responsive to the anti-inflammatory effect of melanocortins express the MC1 receptor, which is the receptor with the greatest affinity for  $\alpha$ -MSH. This receptor subtype expression occurs in monocytes and macrophages, neutrophils, mast cells, fibroblasts, dendritic cells, astrocytes, and microglia (de Angelis et al., 1995; Star et al., 1995; Catania et al., 1996; Rajora et al., 1996; Hartmeyer et al., 1997; Artuc et al., 1999; Becher et al., 1999; Bohm et al., 1999a; Chakraborty et al., 1999; Luger et al., 2000; Scott et al., 2002). Thus, the MC1R likely participates in the anti-inflammatory effects of melanocortin peptides. Furthermore, it appears that MC1R expression can be altered by certain stimuli. Normal human monocytes express a small number of MC1R binding sites that are up-regulated when these cells are activated by various agents such as lipopolysaccharide (LPS) or combinations of cytokines (Bhardwaj et al., 1997).

Evidence from experiments using MC1R-selective synthetic analogs and from immunoneutralization studies supports the idea that MC1R activation contributes to the anti-inflammatory influences of melanocortins. Two MC1R-selective agonists, MS05 and MS09 (Szardenings et al., 2000), down-regulated expression and secretion of endothelial cell selectin (E-selectin), vascular cell adhesion molecule (VCAM), and intercellular adhesion molecule (ICAM), in human dermal vascular endothelial cells treated with tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) (Brzoska et al., 1999a). Furthermore, both MS05 and the MS09 inhibited TNF- $\alpha$ -induced activation of NF- $\kappa$ B in endothelial cells. TNF- $\alpha$  production was likewise reduced by the octapeptide 154N-5, which is an MC1R-specific agonist (Ignar et al., 2003).

Experiments on immunoneutralization of the MC1R subtype in the human monocytic cell line THP-1 (Tahezadeh et al., 1999) provide further support for the idea that the MC1R is significant in immunomodulatory effects of  $\alpha$ -MSH. Receptor neutralization with a specific antibody increased basal and LPS-stimulated production of TNF- $\alpha$  by THP-1 cells. Furthermore, preincubation of cells with the anti-MC1R antibody prevented the inhibitory influences of synthetic  $\alpha$ -MSH on TNF- $\alpha$  production (Tahezadeh et al., 1999). Finally, mice carrying the dominant agouti allele lethal yellow showed greater increases in circulating interleukin 6 (IL-6) after injection of LPS relative to control animals (Lipton et al., 1999). Therefore, hypersecretion of the MC1R antagonist protein agouti enhances the acute inflammatory response to challenge.

In addition to MC1R, there is evidence that other MCR subtypes are involved in the anti-inflammatory effects of melanocortin peptides. Expression of MC3R occurs in murine (Getting et al., 1999, 2001) and human (Tahezadeh et al., 1999) macrophages. Natural and synthetic ligands for this receptor subtype had beneficial

influences in murine urate crystal-induced peritonitis and in experimental gout (Getting et al., 2001, 2002). Systemic treatment with  $\gamma_2$ -MSH in mice with urate crystal-induced peritonitis inhibited accumulation of KC/IL-8, IL-1 $\beta$ , and neutrophils in the peritoneal cavity (Getting et al., 2001). The mixed MC3/4R antagonist SHU9119 (Hruby et al., 1995) prevented the inhibitory actions of  $\gamma_2$ -MSH, whereas the selective MC4R antagonist HS024 (Kask et al., 1998) had no effect. Gouty arthritis induced in rats by monosodium urate monohydrate injections into rat knee joints was likewise reduced by stimulation of the MC3 receptor subtype (Getting et al., 2002). Finally, data from experiments on coronary ligation in rats show that MC3R participate in the protective effect of melanocortins in myocardial ischemia/reperfusion-induced arrhythmias (Guarini et al., 2002).

Consistent with the idea of multiple receptor involvement in the anti-inflammatory effects of melanocortins, expression of the MC5R subtype was found in human B-lymphocyte (Buggy, 1998), monocyte (Tahezadeh et al., 1999), and mast cell (Artuc et al., 1999) lines and in lymphocytes from rat (Akbulut et al., 2001) and mouse (Taylor and Namba, 2001). Therefore, it appears that several MCR subtypes contribute to the anti-inflammatory effects of melanocortin peptides, perhaps in different physiological or pathological conditions, in different tissues, or at different peptide concentrations.

A still unanswered question regards the cell signaling of the C-terminal tripeptide Lys-Pro-Val,  $\alpha$ -MSH (11–13). This small peptide shares the anti-inflammatory and antipyretic effects of  $\alpha$ -MSH (1–13) (Richards and Lipton, 1984b; Hiltz and Lipton, 1989; Mugridge et al., 1991; Hiltz et al., 1992; Poole et al., 1992; Uehara et al., 1992; Watanabe et al., 1993; Ceriani et al., 1994b; Macaluso et al., 1994; Bhardwaj et al., 1996; Ichiyama et al., 1999c; Luger et al., 1999; Haddad et al., 2001; Mandrika et al., 2001). Furthermore,  $\alpha$ -MSH (11–13) reduced NF- $\kappa$ B translocation to the nucleus much as the full-length  $\alpha$ -MSH (Lipton et al., 1999; Barcellini et al., 2000; Mandrika et al., 2001). However, several observations indicate that this molecule does not compete with  $\alpha$ -MSH for receptors expressed by the B16 mouse melanoma cells (Lyson et al., 1994) and does not recognize any of the known melanocortin receptors (Wikberg et al., 2000; Mandrika et al., 2001; Getting et al., 2003b; Muceniece et al., 2003). Therefore, the cell receptor for Lys-Pro-Val is still unknown.

#### *B. Influence of Melanocortins on Nuclear Factor- $\kappa$ B-Mediated Transcription*

The remarkably broad effects of  $\alpha$ -MSH on inflammatory mediator production were puzzling to researchers until the discovery that the peptide inhibits activation of the nuclear factor- $\kappa$ B (Manna and Aggarwal, 1998; Haycock et al., 1999, 2000; Ichiyama et al., 1999a,b,c,d, 2000b; Gupta et al., 2000; Haddad et al., 2001; Mandrika

et al., 2001; Hassoun et al., 2002). This essential nuclear factor induces transcription of many molecules involved in the inflammatory process: its inhibition has, therefore, broad consequences for mediator production and cell functions. NF- $\kappa$ B is present in virtually all eukaryotic cell types. It consists of homodimers and heterodimers of proteins of the Rel family. The first member of this family to be described and the factor commonly referred to as NF- $\kappa$ B consists of a heterodimer of p50 and p65 subunits. NF- $\kappa$ B is retained in an inactive form in the cytoplasm bound to members of the I $\kappa$ B inhibitory protein family (May and Ghosh, 1998). Phosphorylation of I $\kappa$ B by various agents such as drugs, cytokines, bacterial products, and viruses can cause I $\kappa$ B degradation. Subsequently, the free NF- $\kappa$ B is translocated to the nucleus where it binds to sequences of DNA encoding NF- $\kappa$ B-responsive elements and triggers the transcription of target genes. NF- $\kappa$ B participates in the regulation of hundreds of genes, including those for cytokines, chemokines, growth factors of the hematopoietic system, major histocompatibility system, antiapoptotic factors, and inducible nitric oxide synthase (iNOS). Therefore, the discovery that  $\alpha$ -MSH inhibits NF- $\kappa$ B activation has provided an explanation for the broad effects of the peptide on mediator production. In the monocytic cell line U937,  $\alpha$ -MSH down-regulated NF- $\kappa$ B activation induced by a variety of inflammatory stimuli including TNF, endotoxin, ceramide, and okadaic acid (Manna and Aggarwal, 1998). Suppression of NF- $\kappa$ B translocation occurred through generation of cAMP and activation of PKA (Manna and Aggarwal, 1998). Similar results were obtained in experiments on human glioma cells and whole mouse brains stimulated with lipopolysaccharide.  $\alpha$ -MSH and its C-terminal tripeptide KPV modulated brain inflammation by inhibiting NF- $\kappa$ B activation (Ichiyama et al., 1999d). In both models of central nervous system inflammation, the evidence was consistent with  $\alpha$ -MSH-induced modulation of NF- $\kappa$ B activation by limiting I $\kappa$ B- $\alpha$  degradation. Furthermore,  $\alpha$ -MSH modulated activation of NF- $\kappa$ B in human dermal fibroblasts (Bohm et al., 1999b), endothelial cells (Kalden et al., 1999), keratinocytes (Brzoska et al., 1999b), melanocytes (Haycock et al., 1999), and melanoma cells (Haycock et al., 1999).

Experiments on cells transfected with a plasmid vector encoding  $\alpha$ -MSH indicate that the peptide can inhibit NF- $\kappa$ B activation in an autocrine fashion (Ichiyama et al., 1999a, 2000b). In glioma cells and lung epithelial cells transfected with pCMV-ssMSH, there was reduced I $\kappa$ -B degradation and inhibition of NF- $\kappa$ B activation (Ichiyama et al., 1999a, 2000b). Inhibition of NF- $\kappa$ B by plasmid-expressed  $\alpha$ -MSH was likewise observed in human fetal kidney and cytoskeletal muscle cells (Etemad-Moghadam et al., 2002).

Recent research indicates that  $\alpha$ -MSH inhibits NF- $\kappa$ B activation also in human immunodeficiency virus (HIV)-infected monocytes (Barcellini et al., 2000). Through

binding of its long terminal repeat to NF- $\kappa$ B, HIV replication is linked to the state of activation of infected cells. Stimuli that activate NF- $\kappa$ B enhance HIV production (Feinberg, 1992). In these circumstances, viral RNA increases, and the pattern of expression changes to include the singly spliced and unspliced messenger RNA transcripts encoding virion constituents.  $\alpha$ -MSH reduced NF- $\kappa$ B activation and spliced and unspliced HIV RNA in phorbol 12-myristate 13-acetate-stimulated chronically HIV-infected U1 cells (Barcellini et al., 2000). All these observations suggest that  $\alpha$ -MSH could be a candidate for treatment of pathologic conditions in which activation of NF- $\kappa$ B is prominent.

### C. Melanocortins Modulate Production of Chemical Mediators of Inflammation

Since the initial studies on antipyretic and anti-inflammatory influences of  $\alpha$ -MSH, it has been clear that the peptide inhibits production and action of cytokines and other mediators of inflammation (Catania and Lip-ton, 1993). Inhibitory effects on inflammatory mediator production were observed in experiments in vitro and in vivo. In vitro experiments indicated anti-inflammatory influences both in normal cells and in cells from blood of patients with inflammatory or infectious disorders. Effects in vivo occurred during systemic or localized inflammatory host challenge.

1. *Effects in Vitro.* Monocytic cells are significant targets for the anti-inflammatory effects of  $\alpha$ -MSH.  $\alpha$ -MSH down-regulated CD86, a major T-cell costimulatory molecule, in LPS-stimulated monocytes (Bhardwaj et al., 1997). In human peripheral blood monocytes and cultured human monocytes,  $\alpha$ -MSH increased the production and expression of IL-10 (Bhardwaj et al., 1996). Because IL-10 reduces proinflammatory cytokine production in macrophages, its up-regulation can have anti-inflammatory influences. Research in septic patients showed that addition of small concentrations of  $\alpha$ -MSH to LPS-stimulated whole blood samples inhibited TNF- $\alpha$  and IL-1 $\beta$  production by 30 to 40% (Catania et al., 2000b). Cytokine production in monocytes was inhibited also when production was stimulated by a noninflammatory inducer. Indeed, in experiments on normal peripheral blood mononuclear cells,  $\alpha$ -MSH inhibited the production of IL-1 $\beta$  and TNF- $\alpha$  induced by HIV envelope glycoprotein 120 (gp120) (Catania et al., 1998b). The inhibitory effect of  $\alpha$ -MSH on TNF- $\alpha$  production was also observed in whole blood from HIV-positive patients stimulated with endotoxin (Catania et al., 1998b).

The inhibitory effects of  $\alpha$ -MSH on production of cytokines and other mediators of inflammation observed in blood cells was confirmed in monocyte/macrophage and microglial cell lines. In the THP-1 human monocyte/macrophage cell line,  $\alpha$ -MSH inhibited LPS-stimulated release of TNF- $\alpha$  (Taherzadeh et al., 1999).  $\alpha$ -MSH likewise inhibited nitric oxide production induced by LPS plus interferon  $\gamma$  (IFN- $\gamma$ ) in RAW264.7 mouse macro-



phages (Star et al., 1995; Mandrika et al., 2001). In further experiments, inhibitory effects of  $\alpha$ -MSH (1–13),  $\alpha$ -MSH (11–13), and ACTH (1–24) on production of TNF- $\alpha$ , IL-6, and NO were demonstrated in a murine microglial cell line stimulated with LPS plus IFN- $\gamma$  (Delgado et al., 1998). Another observation from this research was that production of TNF- $\alpha$ , IL-6, and NO was greater in activated microglia after immunoneutralization of endogenous  $\alpha$ -MSH, which appears to be an autocrine anti-inflammatory factor in microglia (Delgado et al., 1998). In related experiments,  $\alpha$ -MSH and KPV inhibited TNF- $\alpha$  and NO production by murine microglia stimulated with  $\beta$ -amyloid, a main actor in development of Alzheimer's disease (Galimberti et al., 1999).

$\alpha$ -MSH influences on mediator production are exerted in other relevant cell types, including endothelial cells.  $\alpha$ -MSH down-regulated LPS-induced expression of adhesion molecules VCAM-1 and E-selectin in the endothelial HMEC-1 cell line (Kalden et al., 1999). Inhibitory influences on cell adhesion molecules can be very beneficial in several forms of inflammation including reperfusion injury. Indeed, enhanced expression of adhesion molecules, which causes recruitment of inflammatory cells, is a common and detrimental consequence of endothelial injury. In other experiments,  $\alpha$ -MSH caused an unexpected enhancement of expression and release of IL-8 in resting HMEC-1 cells (Hartmeyer et al., 1997) and in dermal fibroblasts (Bohm et al., 1999b; Kiss et al., 1999). However, consistent with its anti-inflammatory influences,  $\alpha$ -MSH considerably attenuated IL-8 release observed in dermal fibroblasts stimulated with IL-1 (Bohm et al., 1999b). These apparently puzzling effects of the peptide might be interpreted in terms of state of activation of target cells. It may be that  $\alpha$ -MSH induces production of certain molecules such as IL-8 in resting cells, although it down-regulates their production when cells are activated by inflammatory agents.

Various skin cells are targets for the anti-inflammatory effects of  $\alpha$ -MSH (Luger et al., 1997; Slominski et al., 2000). After incubation with  $\alpha$ -MSH, human keratinocytes increased expression and production of the anti-inflammatory cytokine IL-10 (Redondo et al., 1998). In normal human keratinocytes,  $\alpha$ -MSH and ACTH reduced NF- $\kappa$ B activation and TNF- $\alpha$  production (Moustafa et al., 2002). Furthermore,  $\alpha$ -MSH inhibited TNF- $\alpha$ -stimulated ICAM-1 expression in normal human melanocytes and in melanoma cell lines (Hedley et al., 1998).

**2. Effects in Vivo.** Experiments in vivo confirmed the broad influences of melanocortins on the production of inflammatory mediators. Nitric oxide production was reduced in models of endotoxemia, inflammatory bowel diseases, heart transplantation, and brain inflammation (Abou-Mohamed et al., 1995; Rajora et al., 1997b; Delgado Hernandez et al., 1999; Gatti et al., 2002). This chemical mediator was also reduced in models of reperfusion injury (Chiao et al., 1997, 1998) and liver inflammation (Chiao et al., 1996). TNF- $\alpha$ , a cytokine that is

produced in response to many inflammatory stimuli and participates in tissue damage, was likewise reduced by melanocortin treatment in many conditions, including septic shock, experimental brain inflammation, inflammatory bowel disease, heart transplantation, hemorrhagic shock, and several other localized or systemic inflammatory conditions (Rajora et al., 1997a,b; Altavilla et al., 1998; Delgado Hernandez et al., 1999; Gatti et al., 2002). Chemokine production induced in endotoxin-induced liver inflammation, experimental heart transplantation, and ischemic renal injury was inhibited by systemic treatment with  $\alpha$ -MSH (Chiao et al., 1997). In murine LPS-induced cutaneous vasculitis (local Shwartzman reaction), a single injection of  $\alpha$ -MSH significantly suppressed the sustained expression of vascular E-selectin and VCAM-1. This persistent expression contributes to diapedesis and activation of leukocytes, which subsequently leads to hemorrhagic vascular damage; its inhibition can therefore have beneficial effects (Scholzen et al., 2003).

#### *D. Central Control of Peripheral Inflammation*

It is now clear that signals originating in the central nervous system can influence immune function in the periphery (Sundar et al., 1989; Catania et al., 1991). Because  $\alpha$ -MSH acts centrally to inhibit fever and is also produced within the brain, it was postulated that the peptide could act centrally to modulate inflammation in the periphery.  $\alpha$ -MSH injected into the cerebral ventricles inhibited, in a dose-related fashion, the acute inflammation induced by picryl chloride in the mouse ear (Lipton et al., 1991). The precise mechanism of the anti-inflammatory effect of centrally administered  $\alpha$ -MSH is not known, but the mechanisms and pathways of descending pain-modulating influences may provide a useful framework. Pathways descending from the periaqueductal gray substance and nucleus raphe magnus of the brainstem via the dorsolateral funiculus are known to influence pain signals. It may be that  $\alpha$ -MSH activates similar descending inhibitory influences on the spinal cord, dorsal root ganglion, and sympathetic chain to reduce the neurogenic aspect of inflammation (Fig. 4), perhaps by inhibiting release of agents, such as histamine and substance P, that alter vascular permeability and cause pain. Experiments on the influence of centrally and peripherally administered  $\alpha$ -MSH molecules on inflammation induced in the hind paw of mice with spinal transection support the idea of central neurogenic anti-inflammatory pathways (Macaluso et al., 1994). However, the results also clearly indicate that  $\alpha$ -MSH peptides, particularly the tripeptide  $\alpha$ -MSH (11–13), can act peripherally to inhibit inflammation. Central anti-inflammatory influences of  $\alpha$ -MSH were prevented by systemic (but not central) injection of the nonspecific  $\beta$ -adrenergic receptor blocker propranolol and by administration of a specific  $\beta_2$ -adrenergic receptor antagonist (Macaluso et al., 1994). This observation suggests that a

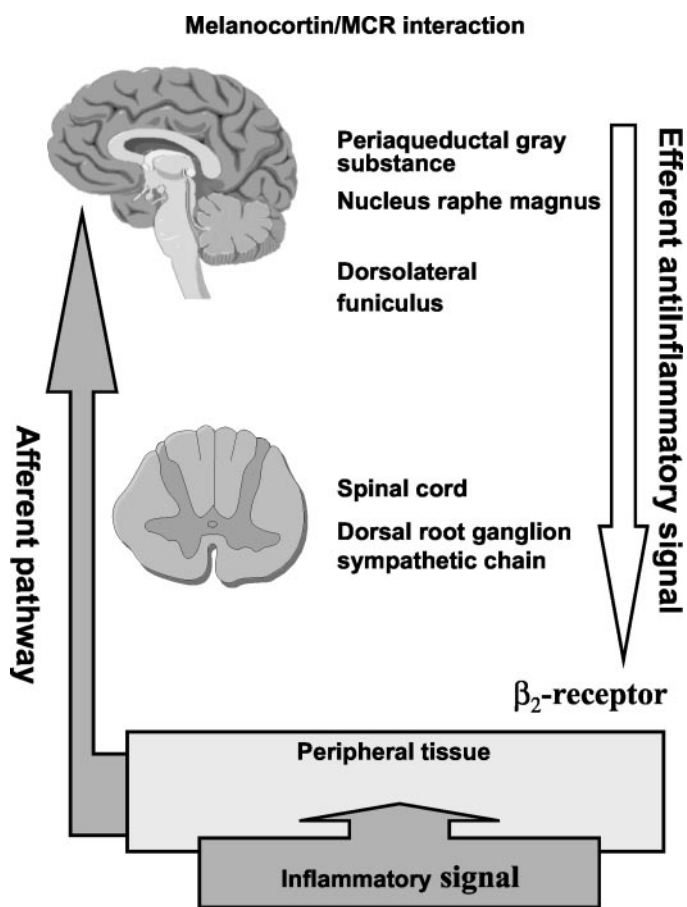


FIG. 4. The precise mechanism of the anti-inflammatory effect of centrally administered  $\alpha$ -MSH is not known, but the mechanism of descending pain-modulating influences may provide a framework. Pathways descending from the periaqueductal gray substance and nucleus raphe magnus of the brainstem via the dorsolateral funiculus to influence pain signals. Perhaps,  $\alpha$ -MSH, after binding to a central MCR, activates similar descending inhibitory influences on the spinal cord, dorsal root ganglion, and sympathetic chain to reduce the neurogenic aspect of inflammation. Central anti-inflammatory influences of  $\alpha$ -MSH are prevented by systemic (but not central) injection of a specific  $\beta_2$ -adrenergic receptor antagonist (Macaluso et al., 1994). Therefore, it appears that a peripheral  $\beta_2$ -adrenergic receptor is required for the central anti-inflammatory effect of  $\alpha$ -MSH.

peripheral  $\beta_2$ -adrenergic receptor is required for the descending anti-inflammatory signal. The effect on acute inflammation was not altered by blockade of cholinergic,  $\alpha$ -adrenergic, or  $\beta_1$ -adrenergic receptors (Macaluso et al., 1994). Edema induced in the mouse ear by intradermal injections of IL-1 $\beta$ , IL-8, leukotriene B $_4$ , and platelet-activating factor was likewise reduced by centrally administered  $\alpha$ -MSH (Ceriani et al., 1994b).

In addition to acute inflammation, the central peptide inhibited host reactions in other conditions of host challenge. Low doses of IL-1 infused into the brain of rats produced rapid suppression of cellular immune responses in lymphocytes isolated from blood and spleen, including natural killer cell activity, response to phytohemagglutinin stimulation, and interleukin 2 production (Sundar et al., 1989). Immunosuppressive effects of central IL-1 were blocked by simultaneous infusion of  $\alpha$ -MSH in the lateral ventricles (Sundar et al., 1989).

Similar results were observed after central administration of HIV envelope protein gp120 (Sundar et al., 1991). Central gp120 induced IL-1 production in the brain and peripheral immunosuppression. When gp120 was infused together with  $\alpha$ -MSH, gp120 no longer decreased natural killer cell activity and lymphoproliferative responses (Sundar et al., 1991). Another effect observed after intracerebral injection of gp120 is enhancement of tumor metastases via centrally produced interleukin-1 (Hodgson et al., 1998). The effect of gp120 on lung retention of tumor cells was blocked by coadministration of central  $\alpha$ -MSH (Hodgson et al., 1998).

Central  $\alpha$ -MSH also reduced systemic host reactions. The peptide injected into the cerebral ventricles of endotoxemic mice reduced iNOS activity and iNOS mRNA in lungs and liver (Delgado Hernandez et al., 1999). Lung myeloperoxidase activity, a marker of neutrophil infiltration, was increased in mice injected with endotoxin, and the increase was significantly less in lungs of animals treated with central  $\alpha$ -MSH (Delgado Hernandez et al., 1999). This research also showed that the endogenous peptide, produced within the brain during systemic inflammation, modulates host responses to endotoxic challenge in peripheral tissues. Indeed, after blockade of central  $\alpha$ -MSH by immunoneutralization proinflammatory agents induced by endotoxin in the circulation, lungs and liver were significantly greater (Delgado Hernandez et al., 1999).

Other melanocortin peptides, in addition to  $\alpha$ -MSH, have central anti-inflammatory influences on peripheral host reactions. Treatment with ACTH (1–24) by the intracerebroventricular route reduced myocardial ischemia/reperfusion injury in rats (Bazzani et al., 2002).

Similar to peripheral influences, central effects of  $\alpha$ -MSH are mediated at least in some part through inhibition of the nuclear transcription factor NF- $\kappa$ B (Ichiyama et al., 2000c). Electrophoretic mobility shift assays of nuclear extracts from murine foot pad injected with TNF- $\alpha$  demonstrated that centrally administered  $\alpha$ -MSH inhibits peripheral NF- $\kappa$ B activation (Ichiyama et al., 1999b). Inhibition of peripheral NF- $\kappa$ B by central  $\alpha$ -MSH is prevented by spinal cord transection and appears to occur via a descending neural pathway (Ichiyama et al., 1999b).

## VII. Antipyretic Influences of Melanocortins

The conception that  $\alpha$ -MSH is important to control host responses stems from the initial observation that the molecule has antipyretic properties (Lipton et al., 1981). This was discovered in a screening for fever-reducing activity of a large number of peptides administered centrally to rabbits made febrile by injection of endogenous pyrogen, which was produced by incubating white blood cells from a donor rabbit with bacterial lipopolysaccharide.  $\alpha$ -MSH reduced fever when given centrally in doses that had no effect on normal body

temperature (Glyn and Lipton, 1981). Neither central nor intravenous injections of antipyretic doses of  $\alpha$ -MSH in rabbits exposed to cold had any effect on their temperature (Richards and Lipton, 1984a). Therefore, like the classic antipyretic drugs, the peptide does not simply inhibit central pathways for heat production and conservation. ACTH (1–24) and ACTH (1–39), which contain the amino acid sequence of  $\alpha$ -MSH and now are known to recognize the same melanocortin receptors, were likewise very potent in reducing fever. Reduction of fever by central or peripheral administration of ACTH in adrenalectomized rabbits indicates that the molecule is antipyretic per se and does not require adrenal mediation (Zimmer and Lipton, 1981).

When it is administered centrally, the antipyretic potency of  $\alpha$ -MSH in reducing a standard fever caused by endogenous pyrogen is very great: more than 25,000-fold greater on a molar basis than that of acetaminophen (Murphy et al., 1983). With intravenous administration the potency of  $\alpha$ -MSH is about 20,000 times that of acetaminophen. The superpotent  $\alpha$ -MSH analog (Nle<sup>4</sup>,D-Phe<sup>7</sup>)- $\alpha$ -MSH, was approximately 10 times more potent than  $\alpha$ -MSH in reducing fever when given centrally (Holdeman and Lipton, 1985). Fevers caused by endotoxin (Martin and Lipton, 1990; Goelst et al., 1991; Villar et al., 1991; Huang et al., 1998) and the cytokines IL-1 (Robertson et al., 1986; Daynes et al., 1987), IL-6 (Martin et al., 1991) and TNF (Martin et al., 1991), but not those caused by IFN- $\alpha$  (Hori et al., 1991) and prostaglandin E<sub>2</sub> (Davidson et al., 1992), were inhibited by  $\alpha$ -MSH. The antipyretic message sequence of  $\alpha$ -MSH (1–13) resides in the C-terminal tripeptide Lys-Pro-Val (Richards and Lipton, 1984b). Although this tripeptide was not as potent as the 1–13 amino acid sequence, it reduced fever when given centrally or peripherally; both acetylated and nonacetylated forms of the tripeptide were effective. N-terminal and intermediate amino acid sequences of  $\alpha$ -MSH have no antipyretic activity. Adding amino acids to the C-terminal tripeptide sequence can either reduce or enhance antipyretic potency (Deeter et al., 1989). Addition of glycine to form  $\alpha$ -MSH (10–13) slightly decreased potency; the 9–13 fragment was almost devoid of antipyretic activity, whereas the potency of the  $\alpha$ -MSH (8–13) sequence was approximately 10-fold greater than that of  $\alpha$ -MSH (11–13) (Deeter et al., 1989).

The great potency of  $\alpha$ -MSH and its presence within the brain suggest that this peptide may be an endogenous modulator of fever. To test this idea,  $\alpha$ -MSH concentration was measured in aliquots of tissue from brains of febrile and afebrile rabbits. During fever,  $\alpha$ -MSH within the septal region increased 2- to 3-fold, whereas there was no significant change in any other region sampled (Samson et al., 1981). This observation was subsequently confirmed in research that showed an increase in septal concentration of  $\alpha$ -MSH during fever but no change in animals with comparably high body

temperature induced by exposure to a hot environment (Holdeman et al., 1985). These observations indicate that the increase in septal concentration of  $\alpha$ -MSH is specific to fever and not caused by nonspecific stress or elevation of brain or body temperature. These findings were interpreted to be compatible with an  $\alpha$ -MSH-specific septal modulation of hypothalamic fever controls. Experiments with push-pull perfusion of the septal region indicated that  $\alpha$ -MSH is released in a pulsatile fashion during endogenous pyrogen-induced fever; such release did not occur when rabbits were afebrile (Bell and Lipton, 1987).  $\alpha$ -MSH release was not dependent on body temperature, and the pulses appeared to result from a direct action of cytokines because some occurred before there was any rise in body temperature. Such pulsatile release is common in neuroendocrine systems, and the peptide released with each pulse would have a certain duration of action, perhaps modulating fever and other host responses. Consistent with this idea, injections of  $\alpha$ -MSH into septal sites reduced fever in rabbits (Glyn-Ballinger et al., 1983; Feng et al., 1987).

The importance of central  $\alpha$ -MSH to control of fever is supported by results of experiments on inactivation of the naturally occurring peptide. After injections of an antiserum highly specific for  $\alpha$ -MSH into the third cerebral ventricle, the febrile response to intravenous administration of endogenous pyrogen was increased somewhat in amplitude but was especially prolonged (Shih et al., 1986). Treatment with  $\alpha$ -MSH antiserum did not alter normal body temperature, nor did injections of normal rabbit serum alter fever. Thus, binding and inactivation of naturally occurring central  $\alpha$ -MSH greatly augment fever but do not affect normal thermoregulation. These observations support the idea that endogenous central  $\alpha$ -MSH is important for fever control, but that it has no role in control of normal temperature. It is notable that rats treated as neonates with injections of monosodium glutamate, which selectively destroys  $\alpha$ -MSH-containing cells in the arcuate nucleus, developed greater increases in temperature after central injections of IL-1 (Opp et al., 1988; Martin et al., 1990) or prostaglandin E<sub>1</sub> (Martin et al., 1990) than did control animals.  $\alpha$ -MSH content of the medial basal hypothalamus and lateral septum of monosodium glutamate-treated rats was reduced relative to control animals (Opp et al., 1988).

Antipyretic effects of central  $\alpha$ -MSH are mediated by MCRs (Huang et al., 1997; Tatro and Sinha, 2003). Intracerebroventricular injection of the synthetic MCR/MC4R antagonist SHU9119 in endotoxin-challenged rats prevented antipyretic influences of  $\alpha$ -MSH. Neither  $\alpha$ -MSH nor SHU9119, alone or in combination, affected body temperatures in afebrile rats. In LPS-treated rats, intracerebroventricular injection of SHU9119 significantly increased fever, whereas intravenous injection of the same dose of SHU9119 had no effect. Neither intracerebroventricular nor intravenous SHU9119 affected



LPS-stimulated plasma ACTH or corticosterone levels. These results indicate that endogenous central melanocortins exert an antipyretic influence during fever by acting on MCRs located within the brain, independent of any modulation of the activity of the pituitary-adrenal axis (Huang et al., 1997).

### VIII. Changes in Endogenous $\alpha$ -Melanocyte-Stimulating Hormone in Inflammatory Disorders

Recognition of changes in endogenous  $\alpha$ -MSH during localized or systemic host reactions has greatly improved understanding of the physiological role of the peptide.  $\alpha$ -MSH is found in plasma, and its concentration in the circulation increased after administration of pyrogens in rabbits (Martin et al., 1989; Martin and Lipton, 1990). In normal human subjects given intravenous endotoxin, there was likewise a fever-related increase in plasma  $\alpha$ -MSH (Catania et al., 1995). Subjects with very high temperatures had marked increases in plasma  $\alpha$ -MSH, whereas those with lower fevers did not. These results suggest that release of  $\alpha$ -MSH is part of the acute phase response to infection in humans and that the molecule rapidly becomes available to modulate host responses in subjects with high fever.

Studies in human disease have shown significant changes of the endogenous peptide in pathological states (Catania et al., 1998a; Ichiyama et al., 2000c). Circulating  $\alpha$ -MSH was increased in plasma of HIV-infected patients relative to matched controls, and its elevation was more pronounced in patients in whom the disease was more advanced (Catania et al., 1993, 1994c). The relationship between concentrations of  $\alpha$ -MSH and disease outcome in HIV infection was explored in a prospective study by Airaghi et al. (1999). This research showed a progressive increment in plasma  $\alpha$ -MSH over time. The novel finding was that marked increases in concentrations of  $\alpha$ -MSH were associated with reduced disease progression or death, whereas patients in whom the peptide remained unchanged over time had a worse outcome (Airaghi et al., 1999). These data suggested that increased concentrations of  $\alpha$ -MSH have a protective influence.

Although in healthy human subjects injected with endotoxin there was a rapid increase in plasma  $\alpha$ -MSH (Catania et al., 1995), concentrations of the peptide were reduced during naturally occurring sepsis syndrome (Catania et al., 2000b). Plasma  $\alpha$ -MSH was low during the critical phase of septic syndrome or septic shock and returned to normal values in patients who recovered but not in those who died (Catania et al., 2000b). Therefore, impaired  $\alpha$ -MSH production had unfavorable prognostic value. Increases in circulating  $\alpha$ -MSH were found in patients with acute myocardial infarction (Airaghi et al., 1995) and in patients on chronic hemodialysis with detectable plasma endotoxin (Airaghi et al., 2000).  $\alpha$ -MSH was found in the synovial fluid of adult patients with

rheumatoid arthritis and young subjects with juvenile chronic arthritis (Catania et al., 1994a,b). Research in children with bacteria and aseptic meningitis showed that cerebrospinal fluid concentrations of  $\alpha$ -MSH were elevated in those with severe bacterial meningitis with neurological sequelae (Ichiyama et al., 2000a).

The negative correlation between circulating  $\alpha$ -MSH and TNF- $\alpha$  early after brain injury and eventual recovery of function found in a recent research is consistent with the idea that reduced  $\alpha$ -MSH production in the brain after injury can have dire consequences (Magnoni et al., 2003). Indeed, the study showed marked reduction in circulating  $\alpha$ -MSH after acute brain injury of either traumatic or vascular origin and that patients with the lowest circulating  $\alpha$ -MSH had an unfavorable outcome (Magnoni et al., 2003).

These observations suggest that there is generally an increase in  $\alpha$ -MSH as a compensatory reaction in the presence of inflammation. It is reasonable to believe that whenever such increase does not occur or is insufficient to counteract action of inflammatory mediators, the disease process is more severe. Treatment with  $\alpha$ -MSH or related synthetic peptides might then be helpful for the patient.

### IX. Potential Therapeutic Targets Based on Preclinical Studies in Inflammatory Disorders

Preclinical studies indicate that melanocortin peptides can be useful in treatment of localized and systemic inflammatory disorders (Lipton and Catania, 1997; Catania et al., 2000a, 2003; Getting and Perretti, 2000; Gantz and Fong, 2003; Luger et al., 2003; Skottner et al., 2003; Starowicz and Przewlocka, 2003). The main potential therapeutic targets are listed in Table 3.

#### A. Acute Inflammation

**1. Allergic Inflammation.** Early studies on anti-inflammatory influences of  $\alpha$ -MSH (1–13) and (11–13) indicated that these peptides inhibit increases in capillary permeability induced by intradermal injections of histamine or IL-1 in rabbits (Lipton, 1989). Subsequently, anti-inflammatory effects of  $\alpha$ -MSH peptides were confirmed in acute skin inflammation induced by nonspecific irritants and cytokines (Hiltz and Lipton, 1989, 1990; Hiltz et al., 1992; Ceriani et al., 1994b).

$\alpha$ -MSH is a significant regulatory mediator of cutaneous immune responses in vivo (Rheins et al., 1989; Hiltz and Lipton, 1990; Grabbe et al., 1996; Luger et al., 1998, 2000). When applied epicutaneously,  $\alpha$ -MSH inhibited both induction and elicitation of contact hypersensitivity responses in mice (Rheins et al., 1989). Systemically administered  $\alpha$ -MSH likewise promoted induction of hapten-specific tolerance (Grabbe et al., 1996). Regional lymph node cells obtained from  $\alpha$ -MSH-treated mice after re sensitization were unable to produce IL-2 in response to trinitrobenzosulfonic acid. In vivo tolerance

TABLE 3  
*Potential therapeutic targets for melanocortin peptides based on preclinical studies*

Therapeutic target	Preclinical Model	Animal, Peptide	Reference
Acute inflammation	Contact sensitivity	Mouse, $\alpha$ -MSH	Robertson et al., 1988; Rheins et al., 1989; Hiltz and Lipton, 1990; Grabbe et al., 1996; Luger et al., 2000
Allergic inflammation	Monosodium urate monohydrate crystals arthritis	Rat, ACTH, $\gamma_2$ -MSH	Getting et al., 2002
Gouty arthritis	Experimental autoimmune uveoretinitis	Mouse, $\alpha$ -MSH	Taylor et al., 2000
Autoimmunity	Adjuvant arthritis	Rat, $\alpha$ -MSH	Ceriani et al., 1994
Autoimmune uveoretinitis	Dextran sulfate-induced colitis	Mouse, $\alpha$ -MSH	Rajora et al., 1997b; Oktar et al., 2000
Chronic inflammation	LPS-induced brain inflammation	Mouse, $\alpha$ -MSH	Rajora et al., 1997a
Rheumatoid arthritis	Nerve crush	Rat, ACTH (1–39), ACTH (4–9) analog ORG2766, ACTH (4–10), $\alpha$ -MSH	Bijlsma et al., 1983; Van der Zee et al., 1988; van de Meent et al., 1997
Inflammatory bowel disease	Cisplatin neurotoxicity	Rat, ORG2766	Gerritsen van der Hoop et al., 1988
Brain inflammation	Taxol neurotoxicity	Rat, ORG2766	Gerritsen van der Hoop et al., 1994
Neurodegenerative disorders	Streptozocin-induced diabetes	Rat, ORG2766	Hammers et al., 1993; Van der Zee et al., 1989; Bravenboer et al., 1993
Peripheral neuropathies	Cecal ligation and puncture	Mouse, $\alpha$ -MSH	Lipton et al., 1994
Nerve injury	Endotoxemia	Rat, HP-228	Abou-Mohamed et al., 1995
Toxic neuropathy	Endotoxemia	Mouse, $\alpha$ -MSH	Delgado Hernandez et al., 1999
Diabetic neuropathy	Intratracheal instillation of endotoxin	Rat, $\alpha$ -MSH	Lipton et al., 1994
Systemic host reactions	Volume controlled hemorrhagic shock	Rat, ACTH-(1–24), ACTH-(1–18), ACTH-(1–17), ACTH-(1–16), NDP- $\alpha$ -MSH	Bertolini et al., 1986; Altavilla et al., 1998; Guarini et al., 1999
Septic shock	Middle cerebral artery occlusion and reperfusion	Mouse, $\alpha$ -MSH	Huang and Tatro, 2002
Acute respiratory distress syndrome	Renal artery occlusion and reperfusion	Mouse, $\alpha$ -MSH	Chiao et al., 1997; Kwon et al., 2000
Hemorrhagic shock	Ligature of the left anterior descending coronary artery	Rat, ACTH (1–24) $\alpha$ -MSH, $\gamma_1$ -MSH	Guarini et al., 2002
Ischemia and reperfusion injury	Mesenteric ischemia and reperfusion	Rat, $\alpha$ -MSH	Hassoun et al., 2002
Organ transplantation	Heterotopic heart transplantation	Rat, NDP- $\alpha$ -MSH	Gatti et al., 2002

induction by  $\alpha$ -MSH could be abrogated by the administration of an anti-IL-10 antibody at the site of sensitization (Grabbe et al., 1996). These data indicate that  $\alpha$ -MSH, in addition to its suppressive effect on induction and elicitation of contact hypersensitivity, is able to induce hapten-specific tolerance in mice and suggest that such effect is mediated by IL-10 release (Grabbe et al., 1996). Therefore contact hypersensitivity acute inflammatory reactions in the skin could be a potential therapeutic target for  $\alpha$ -MSH peptides.

2. *Autoimmune Uveoretinitis.* Recent research indicates beneficial effects of  $\alpha$ -MSH in autoimmune uveoretinitis. Experimental autoimmune uveoretinitis in mice was suppressed in its severity and incidence in animals injected with primed T cells activated in vitro by antigen-presenting cells and antigen in the presence of  $\alpha$ -MSH. Data showed that  $\alpha$ -MSH converted a population of effector T cells into a population of immunoregulatory T cells. Such effector T cells showed similar TGF- $\beta_1$ , absence of IFN- $\gamma$  or IL-10, and unchanged IL-4 productions relative to inactivated T cells (Taylor et al., 2000; Taylor and Namba, 2001). These immunoregulatory influences suggest that  $\alpha$ -MSH could also have beneficial effects in other autoimmune disorders.

3. *Gouty Arthritis.* In a model of gouty arthritis, monosodium urate monohydrate crystals were administered into rat knee joints either alone or in combination with ACTH or the selective MC3R agonist,  $\gamma_2$ -MSH (Getting et al., 2002). Monosodium urate monohydrate crystals produced a knee joint inflammation that was time-dependent and characterized by white cell influx and cytokine release. Local, but not systemic, ACTH had an anti-inflammatory effect at a dose that did not alter circulating corticosterone. This treatment was also effective in adrenalectomized rats. The MC3R/MC4R antagonist SHU9119 blocked anti-inflammatory actions of ACTH but not the anti-inflammatory action of the selective MC3R agonist  $\gamma_2$ -MSH. This research suggests that targeting MC3R subtype could be useful for clinical management of human gouty arthritis and, possibly, other acute arthritis.

## B. Chronic Inflammatory Diseases

1. *Rheumatoid Arthritis.* Research in patients with rheumatoid arthritis and juvenile chronic arthritis showed that  $\alpha$ -MSH is produced in synovial fluid of patients with these rheumatic disorders (Catania et al., 1994a,b). Therefore, the peptide is likely produced at the site of inflammation to counteract influences of proinflammatory mediators. However, the local concentration of the naturally produced peptide could be inadequate to fully control the disease process. If this is true, administration of pharmacological doses of synthetic peptide could be beneficial to reduce inflammation. Consistent with this idea, treatment with  $\alpha$ -MSH significantly reduced joint pathology in rats with adjuvant-induced arthritis, which is a preclinical model of rheumatoid ar-

thritis (Fig. 5) (Ceriani et al., 1994a). Effectiveness of  $\alpha$ -MSH was similar to that of prednisolone, an established treatment for rheumatoid arthritis. However, whereas in prednisolone-treated animals and in control animals there was a significant weight reduction,  $\alpha$ -MSH-treated animals maintained their weight over the observation period. Therefore, in these experiments treatment with  $\alpha$ -MSH was very effective and devoid of the wasting effects of corticosteroids. These observations also indicate that the beneficial influences of  $\alpha$ -MSH on the disease course in a debilitating inflammatory disorder may prevail over the anorectic effects of the peptide. Protection against weight loss was likewise observed in a model of inflammatory bowel disease (see below).

2. *Inflammatory Bowel Diseases.* The mechanism underlying inflammatory bowel disease remains incomplete, but the importance of inflammatory processes is clear and most pharmacological therapies inhibit inflammation. Because the search for more effective agents with low toxicity continues,  $\alpha$ -MSH was administered to mice with dextran sulfate-induced colitis, a model of inflammatory bowel diseases (Rajora et al., 1997b). The peptide treatment had marked salutary effects; it reduced the appearance of fecal blood by over

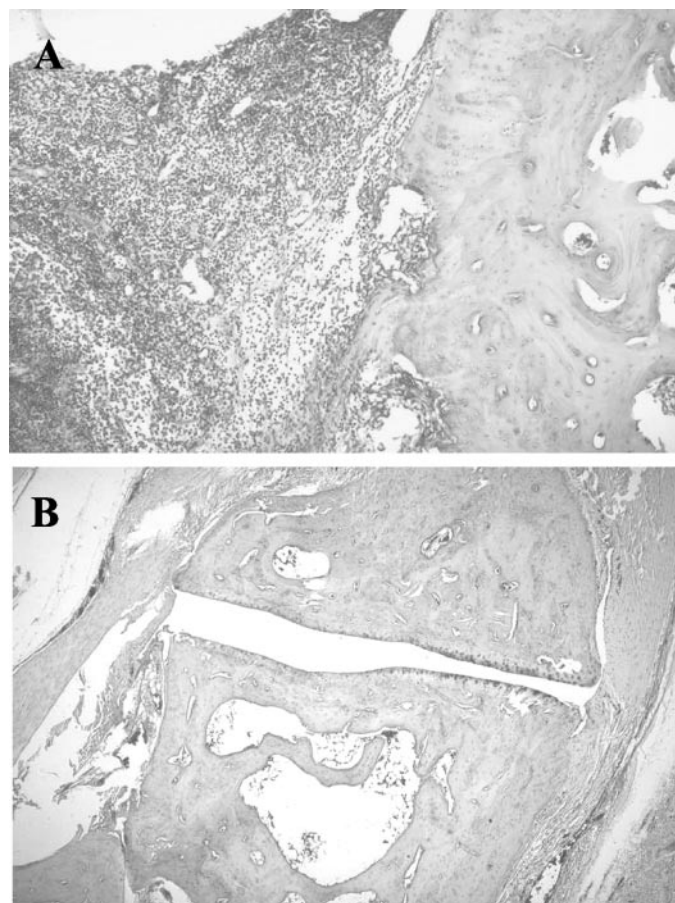


FIG. 5. Effect of systemic treatment with  $\alpha$ -MSH in adjuvant-induced arthritis, a rat model of human rheumatoid arthritis. Synovial infiltration by inflammatory cells (A) was greatly reduced by peptide treatment (B). Reproduced from Ceriani et al., 1994, with permission.



80% (Fig. 6), inhibited weight loss, and prevented disintegration of the general condition of the animals. Mice given  $\alpha$ -MSH showed markedly lower production of TNF- $\alpha$  by tissues of the lower colon stimulated with concanavalin A; the inhibitory effect of  $\alpha$ -MSH on production of inflammatory nitric oxide by lower bowel tissue was even greater.

Consistent with these observations,  $\alpha$ -MSH administration reduced the colonic macroscopic lesions in both acute and chronic colitis induced by trinitrobenzenesulfonic acid (Oktar et al., 2000). The salutary effect of  $\alpha$ -MSH on lesions of acute colitis was reversed by pretreatment with the nitric oxide donor sodium nitroprusside or the cyclooxygenase-1 selective antagonist indomethacin. The results of this study show a protective influence of  $\alpha$ -MSH on colonic lesions which probably involves nitric oxide and prostaglandins (Oktar et al., 2000).

$\alpha$ -MSH had beneficial effects on endotoxin-induced intestinal lesions (San et al., 2001).  $\alpha$ -MSH treatment reduced severity of the lesions macroscopically and microscopically, although the protective effect was mainly confined to the distal ileum. The salutary effect probably involved cyclooxygenase-1 derived prostaglandins in that it was reversed by pretreatment with the nonselective cyclooxygenase-1 inhibitor indomethacin.

### C. Inflammation within the Brain and Neurodegenerative Disorders

Pathogenesis of neurodegenerative disorders involves several effector molecules including cytokines, adhesion

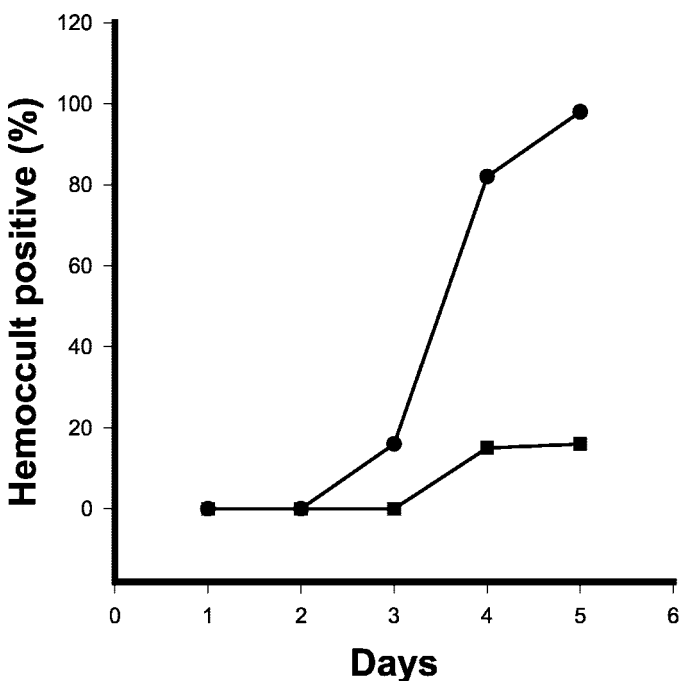


FIG. 6. Influence of  $\alpha$ -MSH treatment in dextran sulfate-induced colitis, an experimental model of inflammatory bowel disease.  $\alpha$ -MSH treatment markedly reduced appearance of occult blood in the stool as shown by proportion of mice with hemocult-positive tests in saline (●) and  $\alpha$ -MSH (■) groups. Reproduced from Rajora et al., 1996, with permission.

molecules, and nitric oxide. One of these molecules, TNF- $\alpha$ , occurs in abundance in lesions of multiple sclerosis and in other neurodegenerative disorders such as Alzheimer's disease. The capacity of TNF- $\alpha$  to promote myelin destruction and increased adhesion molecule expression makes it a prime suspect in etiology of neurodegeneration.

A common feature for several of the mediators involved in neurodegeneration is that their production is under control of the transcription factor NF- $\kappa$ B. Upon certain stimuli such as focal ischemia, glutamate, and hypoxia, there is I $\kappa$ B degradation and free NF- $\kappa$ B is translocated to the nucleus in its activated form. In research on experimental brain trauma, NF- $\kappa$ B was detected in the nucleus of neurons and in microglia of the injured cortex, and its activation persisted over 1 year after injury in glia of the region undergoing atrophy (Nonaka et al., 1999). NF- $\kappa$ B activation likewise occurred in subarachnoid hemorrhage (Ono et al., 1998), and inhibition of this transcription factor had beneficial influences in prevention of vasospasm. Indeed, NF- $\kappa$ B decoy oligo-DNA injected into the subarachnoid space inhibited cerebral vasospasm and morphological changes in vessel walls in a rabbit model for subarachnoid hemorrhage (Ono et al., 1998).

As stated above,  $\alpha$ -MSH is a potent inhibitor of NF- $\kappa$ B activation within the brain (Ichiyama et al., 1999c,d). Studies in experimental brain inflammation showed that  $\alpha$ -MSH protects the inhibitory protein I $\kappa$ B- $\alpha$  from LPS-induced degradation and reduces NF- $\kappa$ B activation and translocation to the nucleus (Ichiyama et al., 1999d). These effects were observed after either central or peripheral injections of the peptide and in vitro (Ichiyama et al., 1999c,d).

The idea that  $\alpha$ -MSH and other melanocortins can have protective influences in the brain is supported by the observation that  $\alpha$ -MSH administration prevented damage in brainstem ischemia and reperfusion injury (Huh et al., 1997). In research on a murine model of stroke, systemically administered  $\alpha$ -MSH likewise exerted neuroprotective effects in cerebral ischemia (Huang and Tatro, 2002). This study showed that  $\alpha$ -MSH reduced intracerebral TNF- $\alpha$  and IL-1 $\beta$  gene expression after transient unilateral occlusion and reperfusion. It appears, therefore, that inhibition of NF- $\kappa$ B-mediated transcription by melanocortins could have beneficial influences against neurodegeneration, in particular after brain ischemia or acute injury.

### D. Peripheral Neuropathies

Many preclinical studies indicate that melanocortins have neurotrophic effects on peripheral nerves.  $\alpha$ -MSH and short peptide sequences of ACTH (1–39); the ACTH (4–9) analog Org 2766, ACTH (4–10), and its analog BIM 22015 are the peptides for which effects on nerve regeneration are better documented (Bijlsma et al., 1983; Edwards et al., 1984, 1986; Verhaagen et al., 1987;

Dekker, 1988; Van der Zee et al., 1988; Strand et al., 1989, 1993, 1994; Antonawich et al., 1994; Hol et al., 1994; Laquerriere et al., 1994; Plantinga et al., 1995; van de Meent et al., 1997; Joosten et al., 1999; Lankhorst et al., 1999, 2000; van der Kraan et al., 1999). A recent study shows that also melanotan II has potent influences in nerve regeneration and neuroprotection (Ter Laak et al., 2003).

In initial studies, slices of fetal spinal cords in culture were used to test effects of  $\alpha$ -MSH and ACTH (4–10) on the outgrowth of neurites from spinal neurons (Van der Neut et al., 1988). These experiments demonstrated that both peptides stimulated axonal outgrowth from fetal spinal cord slices in vitro in dose-dependent way. In subsequent experiments,  $\alpha$ -MSH promoted sprouting and neurite elongation from dissociated rat spinal and sensory neurons (van der Neut et al., 1992). Melanocortin peptides also increased regenerative capacity of peripheral nervous system in postlesion repair. Adrenalectomized rats subjected to sciatic nerve denervation recovered sooner when treated with ACTH (1–39) (Strand and Kung, 1980). Sciatic nerve recovery after crush lesion was likewise accelerated by ACTH (4–10), ACTH (4–9) and its analog ORG2766, and  $\alpha$ -MSH (Bijlsma et al., 1983; Van der Zee et al., 1988). Histological and functional studies suggest that melanocortins do not enhance the rate of outgrowth, but they rather increase the number of newly formed sprouts at the site of lesion (Bar et al., 1993). More recent research suggests that neurotrophic effects induced by melanocortin are mediated by the MC4 receptor subtype (Adan et al., 1996). Indeed,  $\alpha$ -MSH and [D-Phe<sup>7</sup>]ACTH (4–10) were shown to stimulate neurite outgrowth in the neuroblastoma cell line Neuro 2A that expressed the MC4R. Because the MC4R antagonist [D-Arg<sup>8</sup>]ACTH (4–10), inhibited such effect, it is likely that stimulation of neurite outgrowth by  $\alpha$ -MSH was mediated by MC4R (Adan et al., 1996). Beneficial effects of  $\alpha$ -MSH on clinical and neurophysiological recovery after experimental spinal cord injury were demonstrated in a weight drop model of traumatic spinal cord injury in rats (van de Meent et al., 1997).  $\alpha$ -MSH significantly improved recovery, as illustrated by Tarlov scores, thoracolumbar height, and amplitude of rubrospinal motor-evoked potentials. The magnitude of  $\alpha$ -MSH effect on motor performance was comparable with the one observed after treatment with methylprednisolone (van de Meent et al., 1997).

Because of their neurotrophic effects in vivo and in vitro, melanocortin peptides have been tested in animal models of diabetic neuropathy. In streptozocin-induced diabetes, treatment with the ACTH (4–9) analog ORG2766, given during developing neuropathy, gradually restored sensory nerve conduction velocity and motor nerve conduction velocity (Van der Zee et al., 1989). ORG2766 likewise improved already existing neuropathy in streptozocin-diabetic rats (Bravenboer et al., 1993). Beneficial effects observed in preclinical experi-

ments were only partly confirmed in a subsequent clinical investigation (Bravenboer et al., 1994). After 1 year of treatment with ORG2766, there was a significant improvement in vibration threshold, but no other parameters improved in the study period (Bravenboer et al., 1994). There are no data on full-length  $\alpha$ -MSH (1–13) or other synthetic melanocortins that contain the C-terminal tripeptide Lys-Pro-Val in diabetic neuropathy.

Melanocortin peptides may be useful in protecting nerves from damage induced by toxins. In a rat model of cisplatin neuropathy, concomitant administration of ORG2766 protected from cisplatin-induced decrease in nerve conduction velocity (Gerritsen van der Hoop et al., 1988, 1994). Several other preclinical studies confirmed neuroprotective influences of ORG2766 against cisplatin neurotoxicity (de Koning et al., 1987; Gandara et al., 1991; Gispen et al., 1992; Hamers et al., 1992; Bruinink and Birchler, 1993; Cavaletti et al., 1996). Furthermore, treatments with  $\alpha$ -MSH and ORG2766 protected against cisplatin-induced ototoxicity (Heijmen et al., 1999).

Taxol is another highly effective anticancer agent that causes neurotoxicity. Taxol-induced decrease in sensory nerve conduction velocity in rats was prevented by concomitant administration of ORG2766 (Hamers et al., 1993).

Based on evidence from preclinical studies, neuroprotective effects of ORG2766 were assessed in a randomized, double-blind, placebo-controlled trial in women with ovarian cancer treated with cisplatin and cyclophosphamide. ORG2766 attenuated cisplatin neuropathy (van der Hoop et al., 1990). In another clinical trial on men with testicular cancer treated with cisplatin, ORG2766 did not completely prevent cisplatin neuropathy, but nerve damage was ameliorated by the use of this ACTH (4–9) analog (van Gerven et al., 1994).

### E. Systemic Host Reactions

**1. Septic Shock.** Initial experiments on  $\alpha$ -MSH in modulation of host reactions demonstrated that the peptide reduces fever and the acute phase response to endotoxin (Martin and Lipton, 1990). Subsequently, it became clear that  $\alpha$ -MSH peptides given centrally or peripherally modulate many aspects of the host response to endotoxemia (Delgado Hernandez et al., 1999) and improve survival in models of septic shock (Lipton et al., 1994; Abou-Mohamed et al., 1995). Treatment with  $\alpha$ -MSH increased survival in murine septic peritonitis induced by cecal ligation and puncture (Lipton et al., 1994). In untreated animals survival at 24 h was approximately 10%. Survival rose to 50% in animals treated with  $\alpha$ -MSH alone and to 80% when the peptide was associated with the antibiotic gentamicin. Gentamicin alone increased survival to only 40%.

An heptapeptide analog of  $\alpha$ -MSH (HP-228) likewise protected rats against effects of endotoxin (Abou-Mo-

hamed et al., 1995). LPS increased iNOS activity in aortic segments, and HP-228 pretreatment markedly reduced this response. Furthermore, the rate of conversion of arginine to citrulline in lung homogenates from HP-228-treated rats was significantly reduced (Abou-Mohamed et al., 1995).

In a model of acute hepatitis accompanying endotoxemia, hepatic inflammation was induced by administering endotoxin (LPS) to mice pretreated with *Corynebacterium parvum* (Chiao et al., 1996).  $\alpha$ -MSH treatment clearly prevented liver inflammation. The peptide inhibited systemic NO production, hepatic neutrophil infiltration, and increased hepatic mRNA abundance for TNF- $\alpha$ , and the neutrophil and monocyte chemokines (KC/IL-8 and MCP-1). Therefore,  $\alpha$ -MSH prevents LPS-induced hepatic inflammation by inhibiting production of chemoattractant chemokines that cause infiltration of inflammatory cells (Chiao et al., 1996).

2. *Systemic Vasculitis*. Persistent expression of vascular adhesion molecules contributes to enhanced diapedesis and activation of leukocytes, which subsequently leads to hemorrhagic vascular damage in Shwartzman reaction. In murine LPS-induced cutaneous vasculitis, a single injection of  $\alpha$ -MSH significantly suppressed the deleterious vascular damage and hemorrhage by inhibiting the sustained expression of vascular E-selectin and VCAM-1. These observations indicate that  $\alpha$ -MSH may have therapeutical potential for the treatment of vasculitis (Scholzen et al., 2003).

3. *Acute Respiratory Distress Syndrome*. The lung reacts in a similar fashion to various types of acute injury, regardless of etiology. After acute lung injury caused by a variety of infectious or toxic insults, there is diffuse alveolar damage. Acute respiratory distress syndrome is a severe clinical syndrome with a high mortality rate that accompanies diffuse alveolar damage. The disorder is marked by increased vascular permeability of the lung to white blood cells. In a rat model of acute respiratory distress syndrome induced by endotracheal instillation of endotoxin, systemic treatment with  $\alpha$ -MSH greatly reduced leukocyte concentration in the bronchoalveolar lavage fluid (Lipton et al., 1994). Therefore,  $\alpha$ -MSH can reduce white blood cell migration in experimental lung injury.

4. *Hemorrhagic Shock*. Melanocortin peptides also have protective influences in hemorrhagic shock (Bertolini et al., 1986; Altavilla et al., 1998). ACTH fragments or analogs increased blood pressure and pulse amplitude in rats with hypovolemic shock produced by withdrawing about 50% of the estimated total blood volume. The blood and pulse pressure increases remained sustained until the end of the 2-h recording, whereas animals treated with saline died in approximately 20 min. The most active peptide was ACTH (1–24), followed by ACTH (1–16), [Nle<sup>4</sup>,D-Phe<sup>7</sup>]- $\alpha$ -MSH, ACTH (1–18), and ACTH (1–17). These data show that melanocortins reverse otherwise fatal hypovolemic shock and suggest

that they may form a new therapeutic approach for shock treatment (Bertolini et al., 1986). Related research explored the influence of ACTH (1–24) on the blood concentrations of TNF- $\alpha$  in hemorrhage-shocked rats. Circulating TNF- $\alpha$  increased substantially after bleeding in saline-treated rats, whereas in rats treated with ACTH (1–24) there was a smaller increase in circulating TNF- $\alpha$  associated with a almost complete restoration of cardiovascular function. These results suggest that inhibition of TNF- $\alpha$  overproduction by melanocortins could be a significant component in reversing hemorrhagic shock (Altavilla et al., 1998).

#### F. Ischemia and Reperfusion Injury

Tissue damage that occurs during blood reperfusion after an ischemic period is a serious problem in many vascular disorders and reperfusion procedures. Such damage is caused by migration of inflammatory cells when the blood flow is restored and is the consequence of marked up-regulation of endothelial adhesion molecules that occurs during ischemia.  $\alpha$ -MSH treatment reduced ischemia and reperfusion injury in heart (Bazzani et al., 2001, 2002; Guarini et al., 2002), brain (Huh et al., 1997; Huang and Tatro, 2002), kidney (Chiao et al., 1997, 1998; Kwon et al., 2000; Deng et al., 2001; Jo et al., 2001), and gut (Hassoun et al., 2002).

Both ACTH and NDP- $\alpha$ -MSH reduced consequences of short-term coronary ischemia followed by reperfusion and the damage induced by a permanent coronary occlusion in rats (Bazzani et al., 2001). Ischemia was produced by ligation of the left anterior descending coronary artery. Postischemic reperfusion induced ventricular tachycardia in all saline-treated rats and ventricular fibrillation and death in a high percentage of animals. In rats treated with intravenous ACTH (1–24), there was a significantly dose-dependent reduction in the incidence of arrhythmias and lethality. Treatment with ACTH (1–24) almost completely abolished increase in blood/free radical concentration induced by ischemia and reperfusion. In rats subjected to permanent coronary occlusion, the amount of healthy myocardial tissue in NDP- $\alpha$ -MSH-treated animals was significantly greater than in control rats (Bazzani et al., 2001). The protective effect of ACTH (1–24) against the occurrence of ventricular tachycardia, ventricular fibrillation, and lethality was not affected by adrenalectomy or pretreatment with the receptor antagonists HS014 (MC4R), MS05 (MC1R), and HS059 (MC4R/MC5R) antagonist. Rather, the protective effect was prevented by the MC3R/MC4 receptor antagonist SHU9119. These data in rats suggest that MC3R subtype mediates the protective effect of ACTH (1–24) in myocardial ischemia/reperfusion-induced arrhythmias (Guarini et al., 2002).

Related research indicated that the protective effects of ACTH (1–24) in the same models of reperfusion injury also occur when small concentrations of the peptide are injected into the brain (Bazzani et al., 2002). Treatment



with ACTH (1–24) by the intracerebroventricular route reduced the incidence of ventricular tachycardia ventricular fibrillation, fall in mean arterial blood pressure, and lethality induced by 5-min ligation of the left anterior descending coronary artery. Complete protection occurred with an intracerebroventricular dose 10 times less than that needed by the intravenous route. Therefore, the protective effect of melanocortin peptides against myocardial injury caused by ischemia and reperfusion can occur through an action within the brain (Bazzani et al., 2002). This observation reinforces the idea that damage in peripheral tissues can be reduced by marshaling the anti-inflammatory influence of  $\alpha$ -MSH and related peptides within the brain (Lipton et al., 2000).

$\alpha$ -MSH also reduced ischemic brain damage (Huh et al., 1997). Brainstem auditory evoked potentials (BAEPs) and regional cerebral blood flow were measured during ischemia and for 5 h after reperfusion. During the ischemic period, BAEPs were abolished in all animals within 10 min. With reperfusion, the BAEPs increased to approximately 36% of baseline in the control group, whereas the increase was over 60% in animals that received  $\alpha$ -MSH both before and during ischemia. The improved recovery of BAEPs in animals treated with  $\alpha$ -MSH suggests that the peptide may have neuroprotective effects in brainstem ischemia and reperfusion injury (Huh et al., 1997).

After stroke, an intracerebral inflammatory response develops that may contribute to postischemic central nervous system injury. A recent research shows that  $\alpha$ -MSH treatment abolishes intracerebral proinflammatory cytokine gene expression after transient cerebral ischemia and indicates that systemically administered melanocortins may exert neuroprotective effects in cerebral ischemia (Huang and Tatro, 2002). The study shows that  $\alpha$ -MSH reduced activation of intracerebral TNF- $\alpha$  and IL-1 $\beta$  gene expression after transient unilateral occlusion and reperfusion in mice. Systemic  $\alpha$ -MSH treatment suppressed the increase in ipsilateral TNF- $\alpha$  concentration in the cerebrocortical territory of the middle cerebral artery.  $\alpha$ -MSH treatment also inhibited the marked increases in cortical TNF- $\alpha$  and IL-1 $\beta$  mRNA after middle cerebral artery occlusion and reduced the intracerebral TNF- $\alpha$  protein levels observed after transient global ischemia (Huang and Tatro, 2002).

Several investigations indicate that  $\alpha$ -MSH protects against renal injury after ischemia in mice and rats (Chiao et al., 1997, 1998; Kwon et al., 1999, 2000; Jo et al., 2001). Influences of  $\alpha$ -MSH were explored in a model of bilateral renal ischemia (Chiao et al., 1997).  $\alpha$ -MSH significantly reduced ischemia-induced renal damage, measured by changes in renal histology and plasma blood urea nitrogen and creatinine in mice. Furthermore, the peptide significantly decreased tubule necrosis, neutrophil plugging, and capillary congestion. Even when treatment was delayed 6 h after ischemia,  $\alpha$ -MSH

significantly inhibited renal damage. Peptide treatment was associated with reduction in ischemia-induced increases in mRNA for the murine neutrophil chemokine KC/IL-8.  $\alpha$ -MSH also inhibited induction of mRNA for the adhesion molecule ICAM-1, which is known to be critical in renal ischemic injury. The peptide inhibited nitration of kidney proteins and induction of iNOS (Chiao et al., 1997).

Mesenteric ischemia and reperfusion injury to the intestine is a common and often devastating clinical occurrence for which there are few therapeutic options.  $\alpha$ -MSH had protective influences in the postischemic small intestine (Hassoun et al., 2002). The research analyzed the effects of the peptide on intestinal transit, histology, myeloperoxidase activity, and NF- $\kappa$ B activation after 45 min of superior mesenteric artery occlusion and/or 6 h of reperfusion. Rats subjected to ischemia and reperfusion exhibited markedly depressed intestinal transit, histological evidence of severe injury to the ileum, increased myeloperoxidase activity in ileal cytoplasmic extracts, and biphasic activation of NF- $\kappa$ B in ileal nuclear extracts. In contrast, rats treated with  $\alpha$ -MSH before ischemia and reperfusion had intestinal transit and histological injury scores comparable with those of sham-operated controls. In addition, the  $\alpha$ -MSH-treated rats demonstrated less ischemia and reperfusion-induced activation of intestinal NF- $\kappa$ B and myeloperoxidase activity after prolonged reperfusion. There are indications, therefore, that  $\alpha$ -MSH limits postischemic injury to the rat small intestine (Hassoun et al., 2002). Reperfusion injury appears, at present, to be one of the most promising therapeutic targets for melanocortin peptides.

### G. Organ Transplantation

With the increasing need for organ transplantation and the use of "marginal" organs, novel approaches are sought to increase the efficiency and survival of transplanted tissue. It was postulated that treatment with  $\alpha$ -MSH, which does not cause marked immunosuppression but does reduce reperfusion injury, may protect allografts and prolong their survival. NDP- $\alpha$ -MSH treatment caused a significant increase in allograft survival (Fig. 7) and a marked decrease in leukocyte infiltration (Fig. 8) (Gatti et al., 2002). Expression of molecules such as endothelin 1, chemokines, and adhesion molecules, which are involved in allograft rejection, was significantly inhibited in NDP- $\alpha$ -MSH-treated rats. Therefore, protection of the allograft from early injury with  $\alpha$ -MSH can postpone rejection. Addition of this early protection with the peptide to usual treatment with immunosuppressive agents may improve success of organ transplants (Gatti et al., 2002).

### H. Infections

The rapid emergence of microorganisms resistant to conventional antibiotics has hastened the search for new

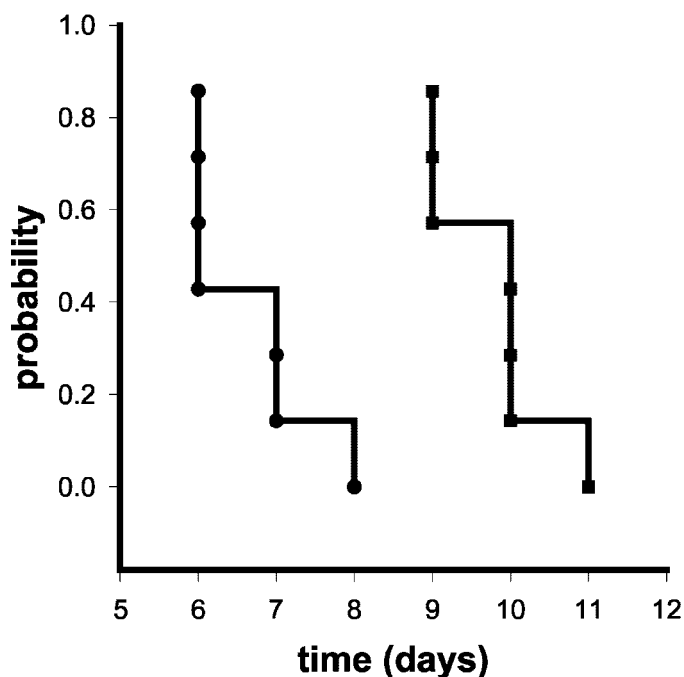


FIG. 7. Effect of NDP- $\alpha$ -MSH treatment on allograft survival in heterotopic heart transplantation in rats. Allograft survival was significantly prolonged in treated rats. ●, untreated; ■, NDP- $\alpha$ -MSH. Reproduced from Gatti et al., 2002, with permission.

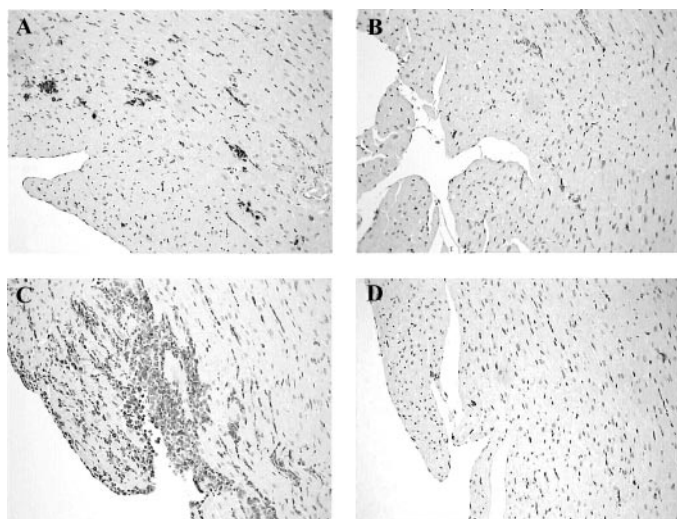


FIG. 8. Histopathology of heart grafts harvested 1 (top) and 4 (bottom) days after transplantation. Sections were stained using the peroxidase-antiperoxidase technique, after incubation with mouse anti-rat ED1 and counterstained with hematoxylin. Heart grafts from untreated rats (A, C) showed diffuse interstitial inflammatory cell infiltration and edema, whereas inflammation and edema were milder and mostly restricted to the subendocardial region in hearts from treated animals (B, D). ED1-positive cells were dense and confluent into microabscesses in untreated animals but fewer and dispersed in hearts of peptide-treated rats. Reproduced from Gatti et al., 2002, with permission.

antimicrobial agents (Boman, 1995; Hancock, 2001). Natural antimicrobial peptides are promising candidates for treatment of resistant bacterial and fungal infections: they kill a broad spectrum of pathogens, and microorganisms resistant to them are uncommon. Most of these peptides are believed to exert their antimicro-

bial activities through either formation of multimeric pores in the lipid bilayer of the cell membrane or interaction with DNA or RNA after penetration into the cell (Hancock, 1997). Antimicrobial influences of  $\alpha$ -MSH were initially explored to test the idea that this established endogenous anti-inflammatory agent could also be anti-infective (Catania et al., 2000c; Cutuli et al., 2000). Indeed,  $\alpha$ -MSH appeared during the Paleozoic era, long before adaptive immunity appeared, and it has similarities with known natural antimicrobial peptides. It is produced by barrier epithelia (Fox and Kraicer, 1981; Thody et al., 1983; Colombo et al., 2002), and it has a positive charge.  $\alpha$ -MSH and its C-terminal tripeptide Lys-Pro-Val were discovered to have potent antimicrobial activity against two representative pathogens, *Staphylococcus aureus* and *Candida albicans* (Cutuli et al., 2000). Evidence suggests that the antimicrobial influences of  $\alpha$ -MSH are exerted through a unique mechanism, substantially different from that of other natural antimicrobial peptides. Indeed, it appears that the candidacidal effect of  $\alpha$ -MSH is linked to the cAMP-inducing activity of the peptide:  $\alpha$ -MSH increased cAMP production in *C. albicans* and the adenylyl cyclase inhibitor dideoxyadenosine partly reversed the candidacidal effect of the peptide (Cutuli et al., 2000).  $\alpha$ -MSH (1–13) and (11–13) not only reduced *Candida* viability, but they also greatly reduced germ tube formation (Fig. 9). The pathogenesis of *C. albicans* infection involves adhesion to host epithelial and endothelial cells and morphologic switching of yeast cells from the ellipsoid blastospore to various filamentous forms: germ tubes, pseudohyphae, and hyphae. It is therefore important that  $\alpha$ -MSH not only reduces *C. albicans* viability but also germ tube

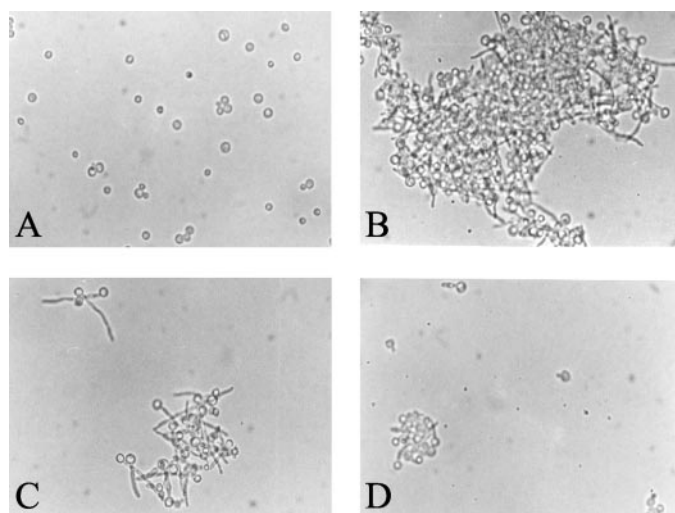


FIG. 9. Influence of  $\alpha$ -MSH peptides on *C. albicans* germ tube formation. A) blastospores; B) horse serum-induced germ tube formation; C) effect of  $\alpha$ -MSH (1–13) treatment on germ tube formation; and D) effect of  $\alpha$ -MSH (11–13) on germ tube formation. The pathogenesis of *C. albicans* infection involves adhesion to host epithelial and endothelial cells and morphologic switching of yeast cells from the blastospores to filamentous forms.  $\alpha$ -MSH not only reduced *C. albicans* viability but also germ tube formation. Reproduced from Cutuli et al., 2000, with permission.

formation. Finally, the research showed that  $\alpha$ -MSH peptides do not reduce killing activity of neutrophils, but they rather enhance it, likely as a consequence of the antimicrobial activity. This characteristic could be very important whenever the peptide is used to treat inflammation in an immunocompromised host.

The C-terminal tripeptide Lys-Pro-Val, which exerts anti-inflammatory influences similar to those of the parent molecule, showed substantial candidacidal influences. A dimer of such a tripeptide, (CKPV)<sub>2</sub>, obtained by inserting a Cys-Cys linker between two units of Lys-Pro-Val-NH<sub>2</sub>, showed excellent candidacidal effects against *Candida spp.* including strains of *C. krusei* and *C. glabrata* (Catania et al., unpublished observations). This molecule is presently in phase I/II clinical trials in the United States and in Europe and should soon be available for clinical use.

Subsequent research explored candidacidal effects of peptides derived from  $\alpha$ -MSH (6–13) with different amino acid substitutions (Grieco et al., 2003). The peptide [D-Nal<sup>7</sup>,Phe<sup>12</sup>]- $\alpha$ -MSH (6–13), which was the most potent of the substituted peptides, reduced viability of *C. albicans* even more effectively than  $\alpha$ -MSH (1–13).  $\alpha$ -MSH peptides that combine antimicrobial, antipyretic, and anti-inflammatory effects could be very useful in treatment of infections.

#### X. Advantages over Currently Used Anti-inflammatory Drugs and Potential Disadvantages

Preclinical investigations indicate that activation of melanocortin receptors could be a novel strategy to control inflammation. As any new therapeutic approach, this strategy may have advantages and potential disadvantages over currently available drugs. The main advantage of melanocortins in the treatment of inflammation is that their influences are broad and are not restricted to a specific mediator or chemical pathway. Indeed, as a consequence of reduced activation of the nuclear factor NF- $\kappa$ B, there is a collective reduction of all the major molecules involved in the inflammatory process.

Another positive feature is that treatment with melanocortin peptides never abolishes the inflammatory response; instead, it modulates it. It is current knowledge that the inflammatory response is a crucial host reaction that contributes to elimination of pathogens and harmful molecules. Cytokines, which are a significant component in the inflammatory process, also have relevant functions in regulation of tissue repair, hematopoiesis, and immune responses. Any agent that completely inhibits their production or action may have detrimental influences to the host defense. Melanocortin peptides modulate enhanced production of cytokines during infection or inflammation but do not abolish their release. Furthermore, they do not affect production of inflamma-

tory mediators in resting conditions. A good example of this is provided by modulatory influences exerted by  $\alpha$ -MSH on the febrile response elicited by pyrogens without any change in nonfebrile body temperature.

A major advantage of melanocortins over currently used anti-inflammatory drugs, in particular corticosteroids, is that the peptides do not reduce microbial killing activity of neutrophils but, rather, enhance it. This characteristic could be very important to treat inflammation in an immunocompromised host.

Lack of selectivity could be a problem connected with the use of natural melanocortin peptides. It is now clear that melanocortins affect many body functions including regulation of food intake, sexual behavior, and pigmentation. Naturally produced peptides likely exert most of their effects through local paracrine and/or autocrine influences, and this may reduce influences at distant targets. Systemic injection of nonselective peptides could, therefore, cause unwanted effects through stimulation of all receptor subtypes. However, design and synthesis of new melanocortin analogs with selective affinity for specific receptors should greatly facilitate targeted effects. Knowledge of amino acid substitutions that reduce binding to each receptor can also help avoid activation of undesired receptor(s). Although recognition of each MCR function is still incomplete, there is general consensus on which receptor subtype(s) should be activated for a specific action. Indeed, current information suggests that MC1R and MC3R should be the targets for synthetic anti-inflammatory melanocortins. Furthermore, local peptide delivery at sites of inflammation, for example in the synovial fluid of an inflamed joint or into a coronary artery during reperfusion, could help convey effects to a specific target. Finally, because certain effects of the melanocortins, such as the anorectic influence, are exerted through activation of MC4R/MC3R within the brain, synthetic ligands that do or do not cross the blood-brain barrier can promote or, rather, avoid activation of these neural receptors. A synthetic anti-inflammatory peptide that does not cross the blood-brain barrier should not reduce food intake.

Another potential problem is that peptide molecules are broken down readily in the circulation or in other body fluids, and natural melanocortins are not an exception. Relatively short half-life could be problematic when sustained blood concentrations are needed. On the other hand, that peptides are not accumulated in the body reduces probability of toxicity and tolerance and allows a better control of pharmacological effects. Peptidomimetic agents targeted to melanocortin receptor subtypes could be more stable and provide sustained blood concentrations. Design and synthesis of such molecules is already in progress, and they could form the basis for novel therapeutic approaches (Haskell-Luevano et al., 1997; Hraby, 2001; Fotsch et al., 2003; Herpin et al., 2003; Mazur et al., 2003; Wikberg et al., 2003).



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